ANTIFUNGAL EFFICACY OF ROUTINE AND NEWER IRRIGANTS ON CANDIDA ALBICANS BIOFILM COLONIZATION IN YOUNG AND OLD HUMAN ROOT CANAL DENTIN - AN EX VIVO STUDY

Dissertation Submitted to

THE TAMILNADU Dr. M.G.R. MEDICAL UNIVERSITY

In Partial Fulfillment for the Degree of

MASTER OF DENTAL SURGERY

BRANCH IV

CONSERVATIVE DENTISTRY AND ENDODONTICS

APRIL 2012

CERTIFICATE

This is to certify that this dissertation titled **"ANTIFUNGAL EFFICACY OF ROUTINE AND NEWER IRRIGANTS ON CANDIDA ALBICANS BIOFILM COLONIZATION IN YOUNG AND OLD HUMAN ROOT CANAL DENTIN - AN EX VIVO STUDY"** is a bonafide record of work done by **DR. ARRVIND VIKRAM** under our guidance during the study period 2009-2012.

This dissertation is submitted to **THE TAMIL NADU DR. M.G.R. MEDICAL UNIVERSITY**, in partial fulfillment for the degree of **MASTER OF DENTAL SURGERY – CONSERVATIVE DENTISTRY AND ENDODONTICS, (BRANCH IV)**. It has not been submitted (partial or full) for the award of any other degree or diploma.

RAGAS DENTAL COLLEGE & HOSPITAL CHENNAI

Guided By:

Dr. R. Indira, MDS
Professor & Head of Dept.
Department of Conservative
Dentistry & Endodontics
Ragas Dental College & Hospital
Chennai

Dr. R. INDIRA
PROFESSOR & HEAD.,
Dept. of Conservative & Endodontics
Ragas Dental College & Hospital,
CHENNAI-600 113

Dr. S. Ramachandran, MDS
Professor & Principal
Department of Conservative
Dentistry & Endodontics
Ragas Dental College & Hospital
Chennai

PRINCIPAL
RAGAS DENTAL COLLEGE & HOSPITAL
CHENNAI

ACKNOWLEDGEMENT

I take this opportunity to express my heartfelt gratitude to my post graduate teacher, mentor and guide ***Dr. R. Indira M.D.S., Professor*** *and* ***HOD,*** *Department of Conservative Dentistry & Endodontics, Ragas Dental College, for her untiring perseverance in motivating and supporting me throughout my postgraduate curriculum. I thank her for her guidance without which this dissertation would not have come true.*

Words seem less to express my deep sense of gratitude to my postgraduate teacher ***Dr. P. Shankar M.D.S.****,* ***Professor,*** *Department of Conservative Dentistry & Endodontics, Ragas Dental College for his invaluable guidance, tireless pursuit for perfection, constant support and encouragement throughout my post graduate curriculum.*

I sincerely thank ***Dr. S. Ramachandran M.D.S., Professor*** *and* ***Principal****, Department of Conservative Dentistry & Endodontics, Ragas Dental College, who immensely supported me during my entire postgraduate curriculum.*

I earnestly thank ***Dr. Anil Kumar M.D.S., Professor*** *and* ***Dr. C.S. Karumaran M.D.S., Professor*** *Department of Conservative & Endodontics, Ragas Dental College, who always helped me with their valuable advice and supported me whenever I was in need.*

I am pleased to thank ***Dr. M. Rajasekaran M.D.S., Associate Professor,*** *Department of Conservative & Endodontics, Ragas Dental College for his constant encouragement throughout the completion of this work.*

I take this opportunity to sincerely thank ***Mrs. Latha, Deputy Head of Microbiology, Lister metropolis ltd, Chennai*** *for assisting me with the analysis of the microbiological experiment. She was extremely helpful, patient and interested throughout the course of the study.*

I am grateful to ***Dr. Malini Thayuman, Senior Scientist, Central research foundation, Chennai*** *for assisting me with my confocal work and helping me get access to use the apparatus. I sincerely thank* ***Dr. Ravanan****, for his guidance with the statistical analysis of this study.*

I would also like to thank ***Dr.B.Veni Ashok****,* ***Dr. A.D. Senthilkumar****,* ***Dr. G. Shankar Narayan****,* ***Dr. S.M. Venkatesan, Dr. Poorni Madan*** *and* ***Dr. D. Duraivel*** *for answering and solving the countless queries that I put to them during the course of my post graduation.*

I will forever remain grateful to my ***batch mates*** *who always inspired me, made me feel at home and made the three years of post graduation a memorable and unforgettable journey.*

*I take this opportunity to thank all my **postgraduate colleagues**, **juniors** and **friends** for their help and good wishes. I earnestly thank the **support staff** and **nurses** of the Department of Conservative Dentistry and Endodontics, Ragas Dental College for helping me during the course of my dissertation.*

*I have reached this far in life only because of the countless sacrifices made by my parents **Mr. V.S Vikram** and **Mrs. Meena Vikram.** I shall always be indebted to them for making me what I am today.*

*Above all else, I am grateful to the **"Almighty"**, who has blessed me with such wonderful people and has given me the opportunity to seek knowledge.*

*I dedicate this dissertation to my grandmother **Mrs. Nirmala Rajasekharan** and late grandparents, **Shri Dr. Rajasekharan Mudaliar** and **Shri Selvaraj Mudaliar**, who always wanted to see me where I am today.*

CONTENTS

LIST OF TABLES

LIST OF GRAPHS

INTRODUCTION

Diseases of the pulp and periradicular tissues are often associated with invasion of Microorganisms. Endodontic infections are known to be polymicrobial in nature with preponderance toward anaerobic species. Kakehashi et al in a classic study proved that bacteria caused pulpal disease; However, in the last decade, numerous studies have revealed a possible role of fungi in the incidence of endodontic infections. Literature reveals that Enterococcus faecalis, Actinomyces, and Candida albicans were the most prevalent microorganisms associated with failed endodontic treatment[25]. There can be no doubt today that microorganisms, either remaining in the root canal space after treatment or re-colonising the filled canal system are the main cause of endodontic failure[14]. The primary endodontic treatment goal must be to optimise root canal disinfection and to prevent reinfection.

Contrary to bacteria, fungi are eukaryotic microorganisms that exhibit two basic structural forms: a ‘‘yeast form’’ (unicellular) and a ‘‘mould form’’ (multicellular). They are common opportunistic pathogens that constitute a part of the normal microbial flora of the oral cavity. Their presence has

been confirmed in dental plaque, dental caries, dentinal tubules, subgingival flora, and infected root canals. The most important fungi belong to genus Candida with C. albicans being the most predominant and commonly isolated yeast from the oral cavity[5].

Candida is a versatile microorganism capable of adapting itself to a wide range of pH levels. It exhibits pleomorphism and grows in many morphologic forms. Furthermore, it exhibits a variety of virulence factors such as ***adherence, hyphal formation, thigmotropism, phenotypic switching and secretes a degenerative enzyme ‘‘aspartyl protease’’ that degrades the dentinal collagen***[35,44]. Candida has the ability to grow on the dentinal surfaces in the absence of oral tissue fluids and penetrates into the dentinal tubules by its various growth patterns (hyphae and blastospores) and by the formation of Biofilms[42]. Sen et al[27] suggested that Candida be considered a ‘‘dentinophilic’’ microorganism as it can invade dental hard tissues and present a reservoir for disseminating candidal infections. Because of these unique qualities, Candida albicans an aerobic microorganism, is able to survive in the harsh ecologic environment of the root canal, which primarily favors the growth of anaerobes .Hence many investigations have confirmed a strong association between persistent or secondary

intraradicular infection with Candida albicans and posttreatment apical periodontitis.

One of the crucial factors for the success of the treatment consists in the eradication of micro-organisms and their by-products from the root canal system. Amongst the procedures involved in the control of endodontic infection, instrumentation and irrigation are important agents in eliminating the micro-organisms from the root canal system. However, mechanical debridement alone does not result in total or permanent reduction of bacteria and fungi . The use of antimicrobial agents in the form of irrigants has been recommended as an adjunct to mechanical instrumentation to reduce the numbers of micro-organisms[1].

Sodium hypochlorite is the most frequently used irrigant in the treatment of infected root canals because of it's antimicrobial, sporicidal, fungicidal, tissue dissolving properties and aids in debridement of canal system. Studies have reported that C.albicans is susceptible to the action of NaOCl with increasing concentration[4].

EDTA was first introduced as a chelating agent in endodontic therapy by Nygaard-Ostby. It reacts with calcium ions in dentin and forms soluble calcium chelate. However EDTA may have an antifungal potential with its chelating property because calcium ions have a critical role in the morphogenesis and pathogenesis of Candida albicans[18,29].

Octenisept is an antiseptic for skin burns, wound disinfection and mouth rinses consisting of octenidine hydrochloride and phenoxyethanol. It demonstrates broad spectrum antimicrobial effects covering both Gram-positive and Gram-negative bacteria, fungi and several viral species[36]. Octenisept has been suggested as a potential endodontic irrigant based on its antimicrobial effects and lower cytotoxicity.

Clotrimazole, a substituted imidazole, is a commonly used antifungal in both medical and dental practice. It is one of a family of azoles and is useful in treating systemic mycoses. They have a broad-spectrum antifungal activity covering the Candida species, dermatophytes, and some gram-positive and anaerobic bacteria such as Staphylococcus aureus and Streptococcus faecalis[43]. However the use of an antifungal

agent as an adjunct in irrigation protocol has been reported only in one study in endodontic literature[25].

Age related histological changes occur in the pulp-dentin complex of teeth[10]. Alterations in dentin tissue with age might result in different adhesion capability of bacteria and fungi. These differences in adhesion of microorganisms and its clinical significance have not been evaluated in the literature. Therefore, the aim of this study was to evaluate the effect of various irrigants on Candida albicans biofilm colonization in Young and Old human root canal dentin by using 2 different techniques: Colony Forming Unit (CFU) method and Confocal Laser Scanning Microscopic (CLSM) method.

The Objectives of this study was to:

1. Investigate the antifungal potential of a new irrigant – Octenisept.
2. Compare Octenisept with Clotrimazole, which is a known antifungal agent.
3. Analyze the adherence capability of C.albicans to Young and Old root canal dentin.
4. Demonstrate the viable and dead fungi in the dentinal tubules using special dyes (SYTO 9 and Propidium Iodide) with the Confocal laser scanning microscope.

REVIEW OF LITERATURE

Sen et al. (1997)[26] discussed that Candida albicans is a fungus that commonly infects human mucosal surfaces. The author added that limited data existed on biofilm formation by Candida albicans on dental surfaces. Human premolar teeth were infected with C. albicans for ten days and hard tissue surfaces were examined with a scanning electron microscope. Enamel, cementum and dentine, in the absence or presence of a smear layer were readily colonized by this microorganism. Hyphae penetrated into cracks, followed the ridges of the cavities and migrated into dentinal tubules. Blastospores and hyphae were embedded in an extracellular material. The author concluded that dental hard tissues may be invaded by Candida albicans and thus can potentially present a reservoir for disseminating candidal infections.

Sen et al. (1997)[27] observed the interaction of Candida albicans with root canal walls and the growth patterns of this microorganism in relation to radicular dentin. Fifteen root sections were infected with Candida albicans and incubated for various periods. The sections were then evaluated by scanning electron microscopy. Blastospores and hyphal structures were

observed on the root canal walls of all specimens. Filamentous hyphal form was dominant in five day specimens. Most of the hyphae and Blastospores showed penetration into dentinal tubules. It was concluded that with the invasive affinity to dentinal structures, Candida albicans may be considered a dentinophilic microorganism.

Sen et al. (1999)[28] evaluated the antifungal properties of 0.12% chlorhexidine, 1% NaOCl and 5% NaOCl. Root sections were enlarged and smear layer was removed in half of the specimens. Each root canal was dispensed with an inoculum of Candida albicans. After 10 days the root sections were treated with 3ml of each disinfecting solution for 1 min, 5 mins, 30 mins and 1 hr. The results revealed that in the presence of the smear layer, antifungal activity was observed only in 1 hour treatment groups for all solutions. However in the absence of smear layer, 5% NaOCl alone started to show antifungal activity after 30 mins. The authors stressed that the antimicrobial effectiveness of irrigating solutions should be re-evaluated particularly in patients predisposed to oral candidiasis.

Baumgartner et al. (2000)[2] evaluated the contents of infected root canals and aspirates of cellulitis/abscesses of endodontic

origin for the presence of Candida albicans using the polymerase chain reaction (PCR). PCR primers specific for the 18S ribosomal RNA gene of Candida albicans were used to survey 24 samples taken from infected root canals and 19 aspirates from periradicular infections of endodontic origins. The presence of C.albicans was detected in 5 of 24 samples taken from the root canals, but none was detected in the periradicular aspirates. The authors concluded that PCR is an extremely sensitive molecular method that may be used to identify C.albicans directly in samples from infections of endodontic origin.

Sen et al. (2000)[29] evaluated the antifungal effect of ethylenediamine - tetraacetic acid (EDTA) on Candida albicans, comparing it with that of various disinfectants and common antifungal agents. The test solutions were sodium hypochlorite, EDTA, chlorhexidine, hexetidine, benzalkonium chloride, povidone-iodine, nystatin and ketoconazole. EDTA was found to demonstrate the highest antifungal activity in comparison with routine antifungal drugs and all other solutions. The authors concluded that EDTA may be strongly recommended during endodontic therapy of patients with a high incidence of oral candidiasis.

Waltimo et al. (2000)[42] developed an in vitro model for investigation of Candida albicans penetration into human dentinal tubules. The authors discussed that the time needed before growth occurred showed great variation with Candida albicans whereas the Enterococcus faecalis model showed penetration within 1 – 5 days of incubation. Slight penetration both by hyphae and yeast cells were observed in specimens inoculated with Candida albicans. The author concluded that penetration of dentin is a possible pathway of infection by Candida albicans.

Valera et al. (2001)[4] evaluated the effect of 1% sodium hypochlorite and five intracanal medications on Candida albicans harvested inside root canals. The contaminated canals were irrigated with sterile saline solution and then treated as follows: 1) filled with Calen paste (calcium hydroxide and glycol polyethylene paste), 2) filled with camphorated paramonochloro phenol (CPMC), 3) Filled with 2% iodine – iodate solution, 4) Filled with tricresol formalin, 5) Filled with Calen and CPMC pastes, 6) Irrigation with 1% sodium hypochlorite and filled with no intracanal medication, 7) No intracanal medication used. The specimens were stored in a humid chamber at 37± 1 ®c for 14 days. The canals were

reinstrumented and sterile paper points were used to transfer the root canal contents to test tubes containing sterile saline solution. Part of the suspension was harvested in Sabouraud dextrose agar with chloramphenicol and incubated for 48 hrs. CPMC was found to be effective in 100% of the samples followed by 1% sodium hypochlorite (70% effective) and calcium hydroxide with CPMC (70% effective).

Ferguson et al. (2002)[7] conducted an in vitro study to determine the susceptibility of the yeast Candida albicans to various intracanal irrigants and medications. The minimum inhibitory concentration (MIC) of sodium hypochlorite, hydrogen peroxide, chlorhexidine digluconate and aqueous calcium hydroxide that is required to kill a standardized inoculum of Candida albicans was determined. Growth of the yeast was measured by optical density. Sodium hypochlorite, hydrogen peroxide and chlorhexidine digluconate were concluded to be effective anticandidal agents with MIC's of <10 µg/ml, 234 µg/ml and 0.63 µg/ml respectively. The authors also added that aqueous calcium hydroxide had no activity.

Siqueira et al. (2002)[33] conducted a study to investigate the pattern of radicular dentin colonization by five fungal species.

Bovine root sections were infected with each of the following fungal species: Candida albicans, Candida glabrata, Candida guilliermondii, Candida parapsilosis and Saccharomyces cerevisiae. After 14 days the sections were examined under scanning electron microscopy. Regardless of the Species, single or budding yeast cells were the only fungal forms observed. Candida albicans colonized most of the specimens. On the other hand, the other four fungal species were found to present discrete or no colonization of the radicular dentin. Candida albicans showed different patterns of dentin infection. Most specimens infected with Candida albicans showed some areas of the root canal walls covered with large colonies of yeast cells and dentinal tubules were heavily infected. The authors concluded that whereas Candida albicans showed the ability to colonize dentin, the other four fungal species did not. This can explain why C.albicans is the fungal species most often found in endodontic infections.

Douglas et al. (2003)[5] examined the pathogenic fungi in the genus Candida and discussed its ability to form biofilms which are found to be resistant to a range of anti fungal agents currently in clinical use including amphotericin B and fluconazole. The author's review also suggests that mixed

biofilms containing Candida and bacterial species have striking interactions between the prokaryotic and eukaryotic cells in these adherent populations.

Lamfon et al. (2003)[13] investigated the ability of Candida albicans to form biofilms on enamel, dentine and denture acrylic of various surface roughnesses. Biofilms of C. albicans were grown on various materials in a constant depth film fermenter and maintained with artificial saliva. Enamel, dentin and denture acrylic were prepared to the same surface roughness using silicon carbide grit. The authors inferred that the total number of yeast cells present on enamel was higher than dentine or acrylic. The highest number of yeast cells were found on the roughest surfaces at 6 hours. Confocal laser scanning microscopy images revealed that the maturation of biofilms on denture acrylic may be dependent upon hyphal cells. It was concluded that both the type of surface and their roughness affect the initial formation and subsequent structure of developing of Candida albicans biofilms.

Sen et al. (2003)[30] developed a reproducible, quantitative model of Candida albicans adhesion to human dentin through the use of a colorimetric method and also evaluated the effect

of smear layer on candidal adhesion. Dentin disks with or without smear layer were incubated with Candida albicans for 4 hours. The disks were then exposed to an (2,3) – bis(2 methoxy – 4 – nitro – 5 – sulphophenyl) – 5 – [(phenylamino) – carbonyl] – 2H – Tetrazolium hydroxide coenzyme Q solution for 2 hours. The colour of the supernatant was determined spectrophotometrically at 492nm. It was concluded that the Formazan assay is a potential microbiological tool for the quantitative determination of Candida albicans to human dentin.

Siqueira et al. (2003)[34] evaluated the effectiveness of four intracanal medications in disinfecting the root dentine of bovine teeth experimentally infected with Candida albicans. Infected dentin cylinders were exposed to calcium hydroxide/ glycerin, calcium hydroxide/ 0.12% chlorhexidine digluconate, calcium hydroxide/ camphorated paramonochloro phenol/ glycerin and 0.12% chlorhexidine gluconate/ zinc oxide. Results indicated that specimens treated with calcium hydroxide/camphorated paramonochloro phenol/ glycerin paste or with CHX/ zinc oxide paste was completely disinfected after one hour of exposure and was concluded to be the most effective in eliminating Candida albicans cells from dental specimens.

Waltimo et al. (2003)[44] aimed to discuss the potential role of yeasts in apical periodontitis and discusses that microbiological reports of apical periodontitis have revealed that yeasts can be isolated in 5 – 20 % of infected root canals. The authors inferred that all isolated yeasts belonged to the genus Candida and the predominant species was found to be Candida albicans. Phenotypic and genotypic profiles showed that they express several virulence factors that are capable of infecting the pulp dentin complex and dentinal tubules. Yeasts were also found to be associated particularly with persistent root canal infections due to the resistance of all Candida species to a commonly used topical medicament – Calcium hydroxide.

Love et al. (2004)[15] discussed the invasion of dentinal tubules by bacteria. He hypothesized that the content and architecture of a dentinal tubule can influence the invasion of Bacteria and Fungi, with tubule patency being important. This can account for regional variations and is particularly seen with dentinal sclerosis, where more advanced sclerotic changes in apical radicular tubules, especially in elderly individuals limit bacterial and fungal invasion in this area. The author also discussed the role of adhesins which mediate the initial interaction of bacteria with dentin and also highlighted its

importance for understanding the development of tubule infection. Recent evidence suggests that Fungal species like Candida albicans and bacteria such as enterococci and streptococci may recognize components present within dentinal tubules such as collagen type 1 which stimulate adhesion and intratubular growth. Specific interactions of other oral bacteria may then facilitate invasion of dentin by select microflora. It is important therefore that the mechanism of invasion and fungal – bacterial interactions are understood to assist development of novel control strategies.

Radcliffe et al. (2004)[22] aimed to determine the resistance of microorganisms associated with refractory endodontic infections to sodium hypochlorite used as a root canal irrigant. Strains of Actinomyces naeslundii , Candida albicans and Enterococcus faecalis were tested against sodium hypochlorite adjusted to 0.5, 1, 2.5 and 5.25 % w/v. Contact times used were 0,10,20,30,60 and 120 seconds. All concentrations of NaOCl lowered cfu below the limit of detection after 10s in the case of A. naeslundii and C. albicans. However E. faecalis proved to be more resistant to NaOCl. It was concluded that the association of E. faecalis with refractory endodontic infection may result at least partially from high resistance of this species to NaOCl.

This was however not the case with Candida albicans and Actinomyces naeslundii.

Siqueira et al. (2004)[35] aimed to throw light on the fascinating role of fungi in endodontic infections. The fungi were found to possess virulence attributes – including adaptability to a variety of environmental conditions, adhesion to a variety of surfaces, production of hydrolytic enzymes, morphological transition and biofilm formation. Fungi have occasionally been found in primary root canal infections, but they seem to occur more often in the root canals of obturated teeth in which treatment has failed. Candida albicans was discussed to be the most commonly isolated fungal species from infected root canals and it was described as a dentinophilic microorganism because of its invasive affinity for dentin. The authors concluded that Candida albicans ability to invade dentinal tubules and resistance to commonly used medicaments was responsible for it being associated with persistent root canal infection.

Jin et al. (2005)[9] assessed the utility of two different labeled lectins - Erythrina cristagalli (ECA) and Canavalia ensiformis (ConA) for visualization of extracellular polymeric substances in Candida albicans. To evaluate the viability of Candidal

biofilms, the authors further studied combination stains SYTO9 and propidium iodide (PI). The latter combination has been successfully used to assess bacterial but not fungal viability although PI alone has been used to stain nuclei in fungal cells. Candida albicans biofilms were developed in a rotating disc biofilm reactor and observed in situ using Confocal scanning laser microscopy. It was concluded that SYTO9 and PI are reliable vital stains that may be used to investigate Candida albicans biofilms and their viability.

Kinney et al. (2005)[12] hypothesized that most fractures occur in teeth that have been altered for example restored or endodontically repaired. It is therefore essential to evaluate the structure and mechanical properties of these altered dentins. One such altered form of dentin is transparent (sclerotic) dentin which forms gradually with aging. The mineral concentration as measured by X - ray computed microtomography was significantly higher in transparent dentin, the elevated concentration being consistent with the closure of the tubule lumens. The authors discussed in detail the change in dentin microstructure and the mineralized collagen scaffolding with age. These changes in root dentin with age assumes clinical

significance when performing root canal disinfection for older teeth as compared to younger teeth.

Mustafa et al. (2005)[18] aimed to study the antifungal effects of calcium – chelating or – binding agents on Candida albicans and compared them with conventional antifungal agents. The tests solutions used were ethylenediamine – tetraacetic acid (EDTA), ethyleneglycol – tetraacetic acid (EGTA), sodium fluoride (NaF), titanium tetrafluoride (TiF4), nystatin and ketoconazole. Minimum inhibitory and fungicidal concentrations of the solutions were determined. The results indicated that EDTA had the highest antifungal and fungicidal activity, followed by titanium tetrafluoride. The authors concluded that EDTA may be recommended as an alternative irrigating solution particularly in persistent root canal infections and in root canals of patients having a high incidence of oral candidosis.

Jill et al. (2006)[8] reviewed the formation of fungal biofilms, especially those of the pathogen Candida albicans. Biofilms are differentiated masses of microbes that form on surfaces and are surrounded by an extracellular matrix. Such infections are particularly serious because biofilm cells are relatively

resistant to many common antifungal agents. Several in vitro models have been used to elucidate the developmental stages and processes required for C.albicans biofilm formation and recent studies have begun to define biofilm genetic control. It is clear that cell-substrate and cell-cell interactions, hyphal differentiation and extracellular matrix production are key steps in biofilm development. Quorum sensing might be an important factor in dispersal of biofilm cells. The authors concluded that the past two years have seen the emergence of several genomic strategies to uncover global events in biofilm formation which may contribute better to our understanding of fungal biofilms and how to tackle them efficiently.

Ruff et al. (2006)[24] compared the antifungal efficacy of 6% sodium hypochlorite(NaOCl), 2% chlorhexidine gluconate (CHX), 17% ethylene diamine tetra acetic acid (EDTA) and Biopure MTAD as a final rinse on Candida albicans in vitro. Specimens of single rooted teeth were randomly divided into four groups. After root canal preparation the teeth were inoculated with Candida albicans and incubated for 72 hrs. The groups were rinsed as follows: 1 ml of 6% NaOCl, 0.2ml of 2% CHX, 1 ml of 17% EDTA and 5 ml of Biopure MTAD. The authors concluded that 6% NaOCl and 2% CHX were equally

effective and statistically superior to Biopure MTAD and 17% EDTA in Antifungal activity.

Sena et al. (2006)[32] investigated the antimicrobial activity of 2.5% and 5.25% sodium hypochlorite and 2.0% chlorhexidine gel and liquid as endodontic irrigating substances against selected single species biofilms. Single species biofilms of Enterococcus faecalis, Staphylococcus aureus, Candida albicans, Prevotella intermedia, Porpyhromonas gingivalis, Porphyromonas endodontalis and Fusobacterium nucleatum were generated and placed on agar medium. The biofilms were then immersed in the endodontic irrigating substances for 30 seconds and also for 5, 10, 15, 30 and 60 minutes, with and without mechanical agitation. It was concluded that mechanical agitation improved the antimicrobial properties of the chemical substances using a biofilm model, favoring the agents in liquid presentation especially 5.25% NaOCl.

Tandjung et al. (2007)[36] aimed to investigate the antimicrobial activity of Octenidine on Enterococcus faecalis ATCC 29212 in a dentin block model. Tooth specimens were infected with E.faecalis for 4 weeks and Octenidine – phenoxyethanol gel (1:1) was applied for different timing: 1 min, 10 min, 7 days

and in a different formula (1:3) for 10 minutes. Octenidine was found to be particularly effective after incubation periods of 10 mins and 7 days. It was concluded that Octenidine was very effective against E.faecalis in dentin disinfection. Further laboratory and clinical studies are required to explore its potential against other endodontic microorganisms.

Brändle et al. (2008)[3] investigated the effects of different growth conditions on the susceptibility of five taxa to alkaline stress. Enterococcus faecalis, Streptococcus sobrinus, Candida albicans, Actinomyces naeslundii and Fusobacterium nucleatum were grown as planktonic cells, allowed to adhere to dentin for 24 hours, grown as monospecies or multispecies biofilms on dentin under anaerobic conditions. Microbial recovery upon direct exposure to saturated calcium hydroxide solution for 10 and 100 minutes was compared with control exposure to physiologic saline. Only E. faecalis and Candida albicans survived in saturated solution for 10 minutes, the latter also for 100 minutes. Planktonic microorganisms were most susceptible. Dentin adhesion was the major factor in improving the resistance of E. faecalis and A. naeslundii to calcium hydroxide whereas the multispecies context in a biofilm was the major factor in promoting the resistance of S. sobrinus to the

disinfectant. In contrast the Candida albicans response to alkaline stress was not influenced by the growth condition.

Turk et al. (2008)[38] observed the colonization pattern of Candida albicans on treated and untreated radicular dentin. Root sections of 10 human mandibular premolars were longitudinally separated into halves. The 20 halves were separated into two groups. In group one, only gross pulpal remnants were removed. Group two on the other hand were treated with a sequential use of 15%EDTA for three minutes and 2.5%NaOCl for three minutes. The specimens were then inoculated with Candida albicans and incubated at 37 degree Celsius for ten days. The authors discussed that Candida albicans was present in all specimens, however the colonization pattern was different. In the untreated group, the main growth pattern was a dense mass forming biofilm layers whereas in the treated group pseudohyphae invaded all root canal surfaces. This fact should be considered when evaluating in vitro Candida Adhesion and/ or Penetration studies.

Kakoli et al. (2009)[10] aimed to determine effects of patients age on the prevalence and depth of bacterial penetration inside the dentinal tubules. Teeth were divided into two groups based

on the patients age (young: 18 – 25 and old: >60), instrumented and inoculated with a standardized bacterial load and incubated. The authors inferred that a significantly higher number of tubules were invaded by bacteria in the young group compared with old group and also the depth of invasion was higher in the younger group. It was concluded that bacterial infection of dentinal tubules occurs to a lesser extent in older patients.

Tirali et al. (2009)[37] tested and compared the in vitro effectiveness of sodium hypochlorite (NaOCl) and Octenidine hydrochloride (Octenisept) at different concentrations in the elimination of resistant microorganisms including S.aureus, E.faecalis and C.albicans over a range of time intervals using a broth dilution method and by assessing the minimum inhibitory concentration of octenisept. The authors ranked the in vitro antimicrobial effect of the most effective concentration of irrigants from strongest to weakest as: 100% Octenisept, 50% Octenisept, 5.25% NaOCl and 2.5% NaOCl. It was concluded that the antimicrobial action is related to type and concentration of irrigant and microbial susceptibility.

Turk et al. (2009)[39] investigated the antimicrobial activity of calcium hydroxide (CH) in combination with glycerin,

chlorhexidine gluconate (CHX), cetrimide or distilled water against Enterococcus faecalis and Candida albicans. The CH preparations with glycerin and CHX demonstrated more antifungal activity than the CH preparations with cetrimide and distilled water. The authors discussed that the antimicrobial activity of CH may change with the type of vehicle and against different microorganisms. Enterococcus faecalis was found to be as resistant as Candida albicans to CH preparations.

Barcelos et al. (2010)[1] evaluated the antimicrobial activity of three root canal irrigants against Enterococcus faecalis, Candida albicans and Staphylococcus aureus. The microorganisms were incubated in the presence of citric acid (6 and 10%), EDTA (17%) and NaOCl (0.5, 1, 2.5, 5.25%). Agar diffusion tests were performed and redox indicator Resazurin was used to evaluate the inhibitory effect of the irrigants on the metabolic activity of these microorganisms. The author concluded that most of the irrigation solutions presented effective antimicrobial activity against Candida albicans. It was also inferred that 2.5% and 5.25% NaOCl are microbicides against S.aureus and the metabolic activity of E.faecalis was severely inhibited when the microorganisms were incubated with 17% EDTA.

Donna et al. (2010)[23] evaluated the starvation – survival behavior, growth and recovery in human serum of Candida albicans and compared it with Enterococcus faecalis. The authors discussed that Candida albicans is a common isolate in posttreatment disease usually as a monoinfection of the root filled canal. A factor likely to contribute to its pathogenic potential in posttreatment infection is an ability to endure starvation and use serum as a nutritional source. Varying cell densities of C.albicans and E. faecalis were suspended in 5% human serum or water for 4 – 6 months. Starvation recovery was assessed by addition of 50% serum to starved cells. Cell survival was monitored by periodic removal of aliquots and viable counts. The authors concluded that Candida albicans is well suited for survival in nutrient limited conditions and can use serum as a source of nutrition and for recovery from starvation. These findings were found to parallel the behavior of E.faecalis which possesses a similar capacity for starvation survival and growth in serum, traits that are of likely importance for their participation in posttreatment infection.

Himani et al. (2010)[14] evaluated the antifungal efficacy of 5% doxycycline hydrochloride(Doxy – HCl), and its comparison with the antifungal efficacy of 2.5% sodium hypochlorite, 17%

EDTA and 0.2% chlorhexidine gluconate against Candida albicans in vitro. Tooth specimens were inoculated with a standard strain of Candida albicans and incubated for 72 hours. The specimens were divided into five groups and were irrigated as follows: 2ml of 2.5%NaOCl, 2ml of 17% EDTA, 2ml of 0.2% CHX and 2ml of 5% Doxy – HCl for one minute. The authors concluded that 5% Doxy – HCl had an antifungal efficacy and was statistically significant to 17% EDTA and 2.5% NaOCl.

Ozdemir et al. (2010)[19] aimed to evaluate the effects of ethylenediaminetetraacetic acid (EDTA) and sodium hypochlorite (NaOCl) on Enterococcus faecalis biofilm growth in root canal dentin of young and old individuals. The root canals of extracted young (<30 years) and old (>60 years) single-rooted human teeth were sectioned at the crown and the apical parts. After treatment with 17% EDTA + 2.5% NaOCl, 17% EDTA alone, 2.5% NaOCl alone, or saline, the samples were incubated in E. faecalis suspension for 24 hours. Thereafter, root canal samples were enlarged again with #3 Gates- Glidden burs, and the removed dentin chips were collected and then plated for counting on agar plates as colony-forming units. The author concluded that root canals from elderly population are more susceptible to canal infection.

However, combined application of EDTA and NaOCl significantly reduces the amount of intracanal biofilm.

Romain et al. (2010)[11] demonstrated the presence of yeast and bacterial biofilms on the surface of tracheoesophageal voice prostheses(TVP's) by a double staining technique with confocal laser scanning microscopy(CLSM). Biofilms of 12 removed TVP's were stained with ConA – FITC and propidium iodide for CLSM. Microbial biofilms on the TVP's consisted of bacteria and filamentous cells. Bacterial cells were attached to the filamentous and unicellular yeast cells thus forming a network. The author concluded that CLSM with a double fluorescence staining can be used to demonstrate biofilms of yeasts and bacteria on the surface of TVP's and other substrates.

Saurabh et al. (2010)[25] evaluated the efficacy of 5.25% NaOCl, 2% CHX and 17% EDTA as a final irrigant with and without the inclusion of an antifungal agent (1% Clotrimazole) on Candida albicans. The experimental specimens were divided into two groups. The irrigant group was divided into three subgroups and irrigated with 5.25% NaOCl, 2% CHX and 17% EDTA respectively. The irrigant with antifungal group was

similar to the irrigant group and was additionally irrigated with 1% Clotrimazole. The author discussed that 5.25% NaOCl exhibited superior antifungal efficacy compared to the other irrigants. On inclusion of 1% Clotrimazole, 5.25% NaOCl and 2% CHX showed significantly greater antifungal properties than 17% EDTA with Clotrimazole. It was concluded that 1% of Clotrimazole proved to be effective against Candida albicans when used as a final rinse.

Parmar et al. (2011)[20] aimed to develop a convenient method for the localization and quantification of live and dead bacteria in human ex vivo mineralized dentinal tubules. The roots from human single rooted teeth were infected with E.faecalis and either treated with calcium hydroxide paste or left untreated. Following further incubation, roots were stained with fluorescent DNA binding reagents, washed thoroughly, sectioned and examined by confocal laser scanning microscopy. Bacteria was found to be distributed in the tubules throughout the length of the roots but tubule penetration distance was slightly reduced in the apical sections. It was concluded that fluorescent viability staining is a convenient, accurate and reproducible method for localizing and quantifying live and dead bacteria in human ex vivo mineralized dentinal tubules.

Jingzhi et al. (2011)[16] aimed to develop a standardized model for quantification of the effectiveness of dentin disinfection by different antibacterial solutions including a new root canal irrigant, Qmix. Dentinal tubules from the root canal side in semicylindrical dentin specimens were infected with Enterococcus faecalis by centrifugation of the bacterial suspension into the tubules. The outer side of dentin pieces was closed, and the specimens were subjected to 1-minute and 3-minute exposure to sterile water, 1%, 2%, 6% sodium hypochlorite (NaOCl), 2% chlorhexidine (CHX), and Qmix. Confocal laser scanning microscopy (CLSM) and viability staining were used to quantitatively analyze the proportions of dead and live bacteria inside dentin. The amount of dead cells in dentin increased with increasing NaOCl concentration and time of exposure. Qmix was equally effective in killing bacteria in dentin as 6% NaOCl; more than 40% and 60% of the bacteria were killed by both at 1 minute and 3 minutes, respectively. The authors concluded that Centrifugation helped to create a heavy, evenly distributed infection deep into the dentinal tubules. The new model made it possible to compare the effectiveness of several disinfecting solutions in killing bacteria inside dentin by a noninvasive CLSM method.

MATERIALS AND METHODS

MATERIALS:

1. Freshly extracted 80 single rooted mandibular premolars
2. 0.9% physiologic saline
3. 5.25% NaOCl
4. 17% EDTA
5. Octenisept
6. 1% Clotrimazole
7. Sabouraud dextrose agar
8. Sabouraud dextrose broth
9. Distilled water
10. Phosphate buffer saline
11. Peptone water
12. Live/Dead stain (Baclight Viability Kit): SYTO 9 & Propidium Iodide
13. Fungal strain: Candida albicans (ATCC90028)

ARMAMENTARIUM:

1. Micromotor – straight angle hand piece
2. Diamond disc
3. Gates glidden drills 2&3
4. 2.5ml syringe and 26 gauge needle

5. Tweezer
6. Test tube holder
7. Conical flask
8. Borosilicate glass tubes – 80 numbers
9. Glass slab
10. Cement spatula
11. Petridish
12. Gloves
13. Mouth mask
14. Inoculation loop

SPECIAL EQUIPMENTS:

1. Autoclave
2. Laminar flow chamber
3. Incubator
4. Electronic balance
5. Vortex mixer
6. Ultrasonic water bath
7. Light microscope
8. Hard tissue microtome
9. Confocal laser scanning microscope

PREPARATION OF MEDIA:

1. Sabouraud dextrose broth

Ingredients

1. Special peptone – 10gm/lit
2. Dextrose – 20gm/lit
3. pH - 5.6

Sabouraud dextrose broth was weighed to measure 3gm and dissolved in 100ml of distilled water in conical flask. The conical flask was plugged with cotton and sterilized in autoclave at 121°c for 15 min at 15 lbs pressure

2. Sabouraud dextrose agar

Ingredients

1. Peptone – 10gm/lit
2. Dextrose – 40gm/lit
3. pH - 5.6

Sabouraud dextrose agar was weighed 6.5gm and dissolved in 100ml of distilled water in conical flask. The conical flask was plugged with cotton and heated till the colour change and sterilized in autoclave at 121°c for 15 min at 15 lbs pressure.

PREPARATION OF MEDIA PLATES:

The prepared sterilized Sabouraud dextrose agar was poured into petridishes to a depth of 5mm under the laminar flow chamber. For every 100ml of the medium 6 plates were poured. The poured plates were allowed to solidify and were refrigerated. For every batch of prepared plates one plate served as a sterility check.

METHODOLOGY

Eighty freshly extracted intact single-rooted human mandibular premolars, stored in saline solution at 4°C, were used in this study. Forty teeth were from young subjects (removal for orthodontic reasons) and the remaining forty from older subjects (removal for periodontal reasons). These served as the two groups in the study. The young subjects were in the age group of 12 – 25 years, whereas the older subjects were above the age of 50 years. The teeth were cleaned to render them free from calculus, tissue tags and other debris and kept in 0.2% sodium azide solution for disinfection.

Preparation of dentin disc samples:

The coronal and apical parts of the teeth were cut with a high-speed diamond disk, resulting in a 4-mm-long mid-part of the root sample per tooth and a total of 80 dentin disc samples. Standardization of each root canal was performed by enlarging the canal with #2 Gates-Glidden burs (0.7 mm diameter). Samples were washed thoroughly, sterilized by autoclave at 121°C for 15 minutes, and preincubated at 37°C in brain-heart infusion (BHI) to ensure no microbial contamination[19].

Grouping of Specimens:

In Group I (teeth from young subjects) and Group II (teeth from older subjects), each forty dentin disc samples, were divided into four subgroups having ten dentin samples each and treated with the following irrigants.

Group I (Young group)	**Group II (Old group)**
IA - 2ml of 17% EDTA for one min and 2 ml of 5.25% NaOCl for one min.	IIA - 2ml of 17% EDTA for one min and 2 ml of 5.25% NaOCl for one min.
IB - 2ml of Octenisept for one min.	IIB - 2ml of Octenisept for one min.
IC - 2ml of 17% EDTA for one min and 2 ml of 5.25% NaOCl for one min. A 5ml flush with distilled water. 2ml of 1% Clotrimazole for one min.	IIC - 2ml of 17% EDTA for one min and 2 ml of 5.25% NaOCl for one min. A 5ml flush with distilled water. 2ml of 1% Clotrimazole for one min.
ID - 2ml of sterile phosphate buffer saline for one min.	IID - 2ml of sterile phosphate buffer saline for one min.

Irrigation Regimen:

Each dentin disc sample was carefully removed from the test tube and placed to rest vertically in a petridish. The canal lumen of the dentin disc was irrigated with:

1. **GROUP IA and IIA** - 2ml of 17% EDTA for one minute followed by 2ml of 5.25% NaOCl for one minute.
2. **GROUP IB and IIB** - 2ml of Octenisept for one minute.
3. **GROUP IC and IIC** - 2ml of 17% EDTA for one min and 2 ml of 5.25% NaOCl for one min. A 5ml flush with distilled water. 2ml of 1% Clotrimazole for one min.
4. **GROUP ID and IID** - 2ml of sterile phosphate buffer saline for one min.

Irrigation was done with the help of a 26 gauge syringe. Following this, the set of instruments were sterilized by soaking the tip in alcohol and flaming them in Bunsen flame.

Microbiology procedures:

A suspension of C.albicans was adjusted to 0.5 turbidity on the Mcfarland scale. The sterile canals of 80 experimental dentin disc samples were inoculated with 0.3ml of the adjusted C.albicans suspension, and each sample was individually submerged in Candida albicans suspension in the glass test tube

vials. The samples were incubated at 36°c and 91% humidity for 72 hrs. Every 24 hrs the vials containing the experimental samples were replenished with freshly made suspension of Candida albicans. At 48 hrs aliquots were taken from each sample using a syringe and plated on 4% sabouraud dextrose agar plate to verify the growth of Candida albicans in each sample tube. Out of the 10 dentin disc samples in each subgroup, 8 samples were assessed to analyze the formation of Colony Forming Units (CFU), whilst the remaining 2 samples were assessed to detect the presence of Live/Dead Fungi in the dentinal tubules using the Confocal Laser Scanning Microscope. Hence, a total of 64 dentin disc samples were subjected to the CFU method and 16 dentin disc samples were used for the CLSM method.

CFU Method:

After 72hrs, the 64 samples were removed from the glass test tube vials and rinsed 3 times with 10 mL of sterile PBS. The root canal of each tooth sample was again enlarged with sterile #3 Gates-Glidden burs (0.9 mm diameter), and dentin shavings were collected into 3 mL of sterile PBS. The Gates-Glidden burs were also placed into the test tube to collect dentin shavings that adhered to the bur. All the tubes were

sonicated in an ultrasonic water bath for 10 minutes to dislodge fungi from the burs and dentin shavings and to disperse fungal aggregation. A 1-µm inoculation loop was used to remove aliquots from the suspension prepared from each one of the 64 dentin disc samples and was plated individually on Sabouraud 4% dextrose agar plates. The plates were incubated at 36°c and 91% humidity for 48 hours. After the incubation period, the growth of C. albicans was assessed with light microscopy at 400X. The number of colony forming units (CFUs) of Candida served as a measure of the antifungal activity.

Confocal Laser Scanning Microscopic method:

The 16 samples to be analyzed by the Confocal Laser Scanning Microscope were removed from the glass test tube vials and rinsed 3 times with 10 mL of sterile PBS. The samples were embedded on methyl methacrylate resin blocks and four evenly distributed transverse sections (1 mm thick) were cut from each sample using the Hard tissue microtome. The cut sections were then stained immediately with the SYTO 9 and propidium iodide (PI) reagents which are marketed as the Live/Dead stain (Baclight; Invitrogen Corporation, Carlsbad, CA, USA) and examined under the Confocal Laser Scanning Microscope.

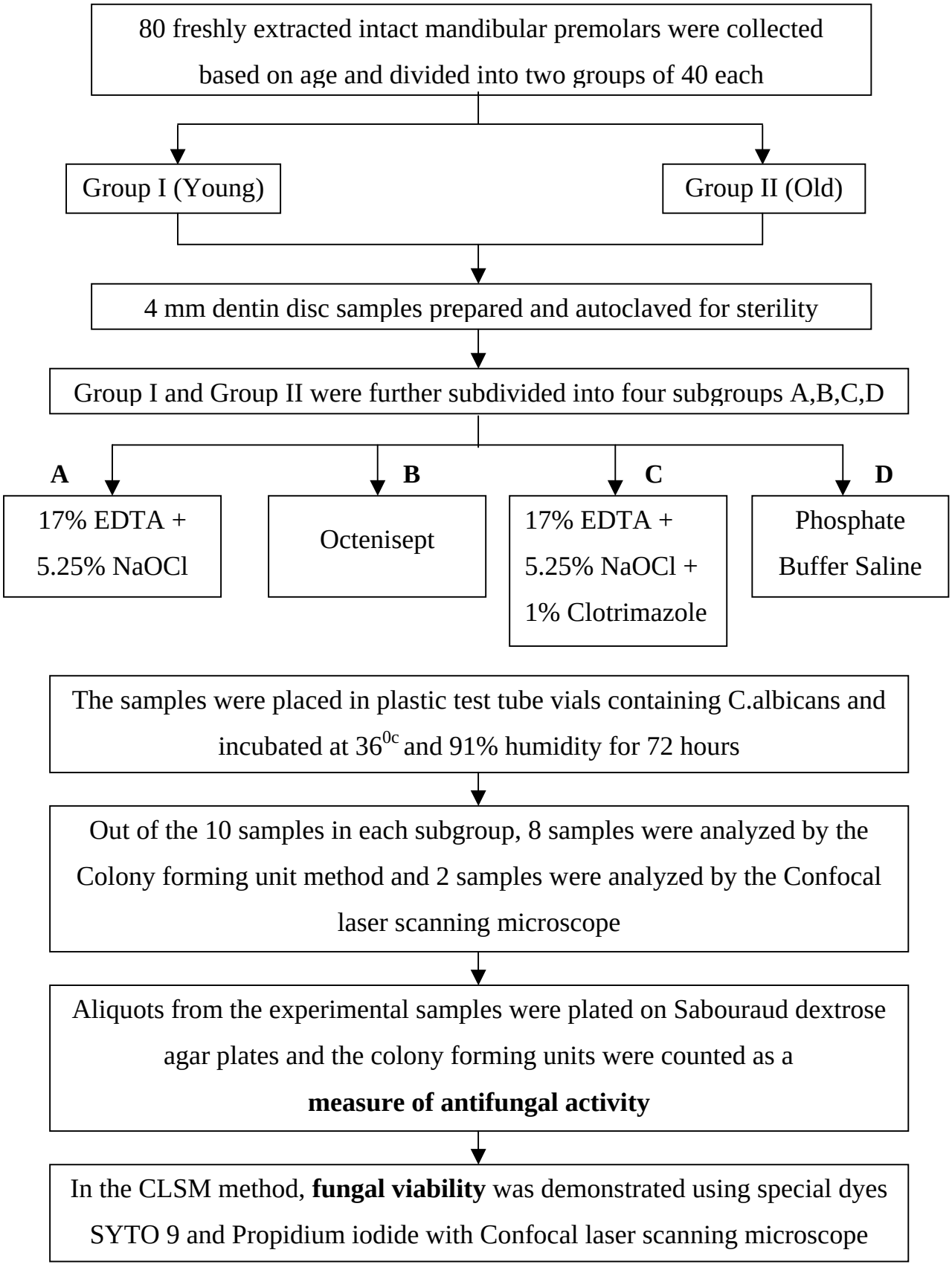
Sections were scanned using Confocal Laser Scanning Microscope (510 META NLO, Axiovert 200; Carl Zeiss Ltd, Jena, Germany) with illumination by a Krypton/Argon laser (488 nm). The border of the root canal was first located with the microscope, and five randomly selected places were scanned with the CLSM for each section. The mounted specimens were observed at 4 different levels of Magnification: 10×, 20×, 40× and 63×. The dimension of each scanned field was 0.70 ×0.70 mm. A 477/543-nm double dichroic mirror was used as an excitation beam splitter and a 545-nm short-pass filter divided green (SYTO 9) and red fluorescence (Propidium Iodide) between the photomultipliers. A 505- to 550-nm band-pass filter was used to visualize SYTO 9 and a 650-nm long-pass filter for PI.

CLSM works on the principle of fluorescence. The advantage of fluorescence for microscopy is that fluorescent dye molecules can be attached to specific parts of any sample, so that only those parts are the ones seen in the microscope. Therefore, it is possible to distinguish two different parts of a particular sample. CLSM has the following parts: Laser source, beam splitter, scanner, objective lens, photomultiplier and a pin hole[45].

However, never is a complete image of the sample formed at any given instant; only one point of the sample is observed. The detector is attached to a computer which builds up the image, one pixel at a time[31].

The Confocal laser scanning microscopic (CLSM) images were recorded in the fluorescent mode. Fluorescence images were analysed with Amira 5.0 (Visage Imaging Inc., Andover, MA, USA), and image stacks were viewed with LSM Image Browser (Carl Zeiss Ltd). The initial stacks, comprising both green and red fluorescence, were split into individual component colour channels and saved as grey-scale images. For each greyscale image, fluorescence was adjusted ('thresholded') such that signals of intensity less than 20% were regarded as background. The split greyscale images were then combined and calibrated to form a single fluorescent image which was qualitatively analyzed by three independent blinded observers to determine the proportion of green and red fluorescence denoting the presence of live and dead Candida albicans cells in the mineralized human dentinal tubules.

Methodology



STATISTICAL ANALYSIS

The results of the present study were subjected to statistical analysis to interpret the significant differences among various irrigants used in the Young and Old groups respectively and also assessed the difference between the groups when the irrigant used was a constant. One-Way ANOVA, *post hoc* Tukey HSD tests and unpaired t-test were used for statistical analysis in the present study.

One-way Analysis Of Variance (ANOVA) is used to study the overall variance within groups. It is the extension of the between groups t-test to the situation in which more than two groups are compared simultaneously. However, it is not possible to identify the difference between the various subgroups with the help of the P values obtained from ANOVA. Therefore a specific statistical test was used for intra-group comparison. Hence, the Tukey HSD is done in order to determine which groups differ from each other. The Tukey Test Honestly Significant Difference or HSD test is a post hoc test designed to perform a pair wise comparison of the means to identify the specific sub groups in which significant differential expression occurs.

Unpaired t-test is applied to unpaired data of independent observation made on individuals of two different or separate groups or samples drawn from two populations.

In this study One way ANOVA followed by Tukey HSD test showed statistically significant difference among various subgroups concerning the discrepancy in the colony forming unit in each group. Unpaired t-test showed a significant difference in the number of colony forming unit between the groups.

TABLE 1

Comparison of the growth of CFUs (1 x $10^3 ml^{-1}$) of Candida albicans in two groups with 4 irrigants

GROUPS	YOUNG – I (Mean±SD)	OLD – II (Mean±SD)
A – 17%EDTA + 5.25%NaOCl	62.25±8.73	185.75±16.36
B - Octenisept	9.38±3.02	38.75±6.23
C – 17%EDTA + 5.25%NaOCl + 1%Clotrimazole	14.88±4.49	51.13±10.87
D – Phosphate buffer saline	570.63±33.02	997.25±74.66
P VALUE:	0.000**	0.000**

Note - ** denote significant at 1% level

TABLE 2

Comparison of growth of CFUs (1 x $10^3 ml^{-1}$) of Candida albicans between the 4 irrigants in each group

GROUPS	YOUNG – I (P Value)	OLD – II (P Value)
A × B	0.000**	0.000**
A × C	0.000**	0.000**
A × D	0.000**	0.000**
B × C	0.919	0.918
B × D	0.000**	0.000**
C × D	0.000**	0.000**

Note - ** denote significant at 1% level

TABLE 3

Comparison of growth of CFUs (1 x $10^3 ml^{-1}$) of Candida albicans for each irrigant sub group between the groups

GROUPS	YOUNG – I (Mean±SD)	OLD – II (Mean±SD)	P Value
A – 17%EDTA + 5.25%NaOCl	62.25±8.73	185.75±16.36	0.000**
B - Octenisept	9.38±3.02	38.75±6.23	0.000**
C – 17%EDTA + 5.25%NaOCl + 1%Clotrimazole	14.88±4.49	51.13±10.87	0.000**
D – Phosphate buffer saline	570.63±33.02	997.25±74.66	0.000**

Note - ** denotes significance at 1% level.

Table 1 demonstrates the mean CFUs of Candida albicans after a final rinse with the irrigant solution in both age groups. Octenisept was found to be effective against Candida albicans followed by 17%EDTA + 5.25%NaOCl + 1%Clotrimazole, 17%EDTA + 5.25%NaOCl and finally, phosphate buffer saline in both Age groups (P<.001). This difference was highly statistically significant.

Table 2 demonstrates the significance of the P value between the four irrigant solutions used in each group (Specific intra group comparison). When Octenisept and 17%EDTA + 5.25%NaOCl + 1%Clotrimazole were compared specifically in both age groups, the difference was not statistically significant (suggesting both irrigant combinations were equally effective). All other comparisons of irrigants within each group were highly statistically significant (P<.001).

Table 3 demonstrates the comparison of mean CFUs of Candida albicans after a final rinse with:

a. 17%EDTA + 5.25%NaOCl in both age groups
b. Octenisept in both age groups
c. 17%EDTA + 5.25%NaOCl + 1%Clotrimazole in both age groups
d. Phosphate buffer saline in both age groups

All the irrigant solutions used in this present study were more effective in the Younger group than the older group. This difference was highly statistically significant (P<.001).

DISCUSSION

Microorganisms play a fundamental role in the etiology of pulp and periapical diseases. Therefore the most important goal of endodontic treatment is the complete debridement of the root canal system to eliminate these entire microorganisms, their by products and tissue debris from the infected root canals. As access to the root canal system is limited and due to its anatomic complexities microorganisms may remain in the dentinal tubules and in other irregular spaces, and when these microorganisms find a supporting environment they can proliferate and reinfect the root canal system. Primary root canal infections are polymicrobial. The most frequently isolated microorganisms in infected root canals include gram –ve anaerobic rods, gram +ve anaerobic cocci , gram +ve anaerobic and facultative rods, lactobacillus species, gram +ve facultative streptococcus species and some fungi[2]. The presence of these microorganisms can result in root canal treatment failures. Various studies in literature over the last decade have reported that the two most common organisms reported to be associated with root canal failure cases are E.faecalis and Candida albicans[3,23,39]. Peciuliene et al (2001)[21] reported C.albicans in 6 of 33 cultures, positive for root filled teeth associated with

periradicular lesions. Molander et al (1998)[17] found C.albicans in 13 of 68 cases of root filled teeth with apical periodontitis. There is a paucity in endodontic literature, targeting the antifungal effects of emerging endodontic irrigants.

Fungi are considered normal inhabitants of the oral cavity, but may produce disease when there are local or immunodeficiency related systemic factors predisposing the individual to infection. Candida albicans is the most encountered commensal and pathological fungi in the oral cavity. Candida can adapt to a wide range of pH, change gene expression in response to environmental conditions, adhere to a variety of substrates, produce degenerative enzymes and change morphological form to evade the host immune system. Candida species have surface molecules that mediate adherence to the host tissue. These molecules include a receptor which binds RGD (Arginine-glycine-aspartic acid) groups on IC_3b, fibrinogen, fibronectin, laminin and vitronectin. Candida species are also able to bind to Collagen type I and IV of dentin and can use dentin as a nutrient source[7,35].

Candida albicans is often described as a dimorphic fungus that exists in blastospore and hyphal forms, nonetheless it is

infact a polymorphic fungus because it has been reported to grow in a number of morphologic forms such as blastospore, germtube, true hyphae, pseudohyphae and chlamydospore depending on the environmental conditions[8]. Sen et al (1997)[27] investigated the growth patterns of C.albicans in relation to human radicular dentin and observed blastospores and hyphal structures on the root canal walls of all specimens. Most of these structures, particularly hyphae exhibited penetration into the dentinal tubules. It was proposed that the contact sensing (Thigmotropism) ability of hyphal structures of Candida made dentinal invasion inevitable. Therefore, on the basis of this invasive affinity to dentin, they considered C.albicans a ***dentinophilic microorganism***. Sen et al (1997)[26] & (2003)[30] demonstrated that the presence of smear layer increased the adhesion of C.albicans to dentin. They hypothesized that this increased adhesion was attributable to the availability of the disintegrated organic structure of dentin and the availability of calcium ions as a source of growth and adhesion.

Fungi can gain access to the root canal through contamination during endodontic therapy and can be involved in the etiology of periradicular lesions[44]. Sundqvist et al have isolated Candida albicans in 6 out of 24 canals where the

endodontic treatment has failed[34]. Baumgartner et al (2000)[2] found Candida albicans in 21% of samples taken from infected root canals while using PCR. Siqueira et al (2004)[35] detected Candida albicans in 2 of 22 patients in case of failed endodontic therapy by PCR.

All these studies prove beyond doubt that C.albicans are the most common yeast isolates found in the root canals of endodontic failure cases. However, investigations on Fungi in recent literature have been limited as opposed to the exhaustive analysis of the facultative anaerobe E.faecalis. Hence, Candida albicans (ATCC 90028) was the test organism chosen in this investigative study.

The prime objective of chemomechanical preparation is the elimination of these organisms. This study design was to investigate the adhesive capability of a microorganism to chemically treated dentin, which could help us to understand how microbes like Candida albicans can attach and survive in a post treatment apical periodontitis milieu.

The antifungal efficacy of sodium hypochlorite was investigated by Radcliffe et al (2004)[22] and reported sodium

hypochlorite in all concentrations (0.5, 1.0, 2.5 and 5.25%) to be effective against Candida albicans in a time period of 10 seconds by agar diffusion method. Estrela et al (2003)[6] by direct exposure test found that NaOCl was effective against C.albicans. However, the agar diffusion method and direct exposure test have been found to have inherent flaws in design. Vianna et al (2004)[41] reported a maximum time for growth inhibition of 30 min for NaOCl used against C.albicans. The search for newer endodontic irrigants with increased antimicrobial efficacy assumes great importance in this scenario.

Sen et al (2000)[29] evaluated the antifungal efficacy of EDTA on C.albicans, comparing it with that of various disinfectants and common antifungal agents and demonstrated that EDTA showed the highest antifungal activity in comparison to all other solutions and antifungal drugs. Mustafa et al (2005)[18] evaluated the antifungal effect of calcium chelating or binding agents on C.albicans comparing with conventional antifungal agents and showed that EDTA had the highest antifungal and fungicidal activity among all the tested solutions.

Tirali et al (2009)[37] compared the in vitro antifungal effectiveness of sodium hypochlorite and Octenisept against Candida albicans by the broth dilution method and demonstrated that Octenidine dihydrochloride was more effective than 5.25% NaOCl solution. It was suggested that ***Octenisept*** solution might be an effective endodontic irrigant. Tandjung et al (2007)[36] investigated the antimicrobial activity of octenidine on Enterococcus faecalis in his study on tooth samples and found that octenidine was particularly effective against E.faecalis in dentin disinfection.

The susceptibility of Candida albicans to different antifungal agents were also evaluated. Waltimo et al (2000)[43] compared the susceptibility of C.albicans to five different antifungal agents (Amphotericin B, 5 Fluorocytosine, Fluconazole, Miconazole and Clotrimazole) and found that Clotrimazole and Fluconazole was most effective against endodontic isolates of C.albicans. Saurabh et al (2010)[25] evaluated the antifungal efficacy of 5.25% NaOCl, 17% EDTA and 2% CHX with and without the addition of 1% Clotrimazole in his study on tooth samples. It was demonstrated that the addition of ***1% Clotrimazole*** proved to be effective against

C.albicans when used as a final rinse following the use of routine endodontic irrigants.

The aim of the present study was to evaluate the effect of universally used routine endodontic irrigants - Sodium hypochlorite and EDTA, newer endodontic irrigants - Octenisept and Clotrimazole on Candida albicans biofilm colonization in Young and Old human root canal dentin by using two different techniques: Colony Forming Unit (CFU) method and Confocal Laser Scanning Microscopic (CLSM) method.

Eighty freshly extracted intact single-rooted human mandibular premolars were selected in the study, forty of which were from young subjects (removal for orthodontic reasons) and the remaining forty from older subjects (removal for periodontal reasons).The teeth were kept in sodium azide solution for disinfection. The young subjects were in the age group of 12 – 25 years, whereas the older subjects were above the age of 50 years. Teeth were collected from young and old subjects to analyze the effect of age on adherence capability of Candida and its potential to colonize the root canal dentin. ***There is only limited knowledge on the effect of dentin aging***

on microbial adhesion, although such information is clinically important. Hence, teeth from younger and older subjects were included in this study, to explore the significance of age in the clinical scenario.

From each tooth, a 4mm long dentin disc sample was prepared which resulted in a total of 80 dentin disc samples. Root canals were standardized with the #2 Gates-Glidden burs (0.7 mm diameter), autoclaved and incubated overnight in BHI medium to ensure no microbial contamination and complete sterility. Following this the samples in the two groups were sub grouped under various experimental irrigants. The irrigants used in the study were 17%EDTA + 5.25%NaOCl, 100% Octenisept, 17% EDTA + 5.25%NaOCl + Clotrimazole and Phosphate buffer saline.

The irrigation regimen used in group IA and IIA was 2ml of 17% EDTA for one minute followed by 2ml of 5.25% NaOCl for one minute. This combination of the universal endodontic irrigants was chosen to simulate the synergistic use of these two agents in routine endodontic regime. 2ml of 17% EDTA was taken in the present study based on the previous studies by Sen et al *(*2000*)*[29], Mustafa et al (2005)[18] who proved the

antifungal efficacy of 17%EDTA on C.albicans. The contact time was for one min as recommended by Ruff et al (2006)[24]. 2ml of 5.25%NaOCl was taken based on the studies by Sen et al (1999)[28] and Sena et al (2006)[32] who demonstrated that 5.25% NaOCl was effective against C.albicans in the absence of smear layer. One minute of contact time was taken in accordance to the previous study done by Radcliff et al[22].

The irrigant of choice in groups IB and IIB was 2ml of 100% Octenisept for one mintute. This irrigant was chosen to explore its antifungal potential based on the study by Tirali et al (2009)[37] who demonstrated that 100% Octenisept is more effective than 5.25% NaOCl as an antimicrobial endodontic irrigant. Previous studies showed the efficacy of octenidine against dental plaque-associated bacteria, such as Streptococcus mutans and Actinomyces viscosus comparable to chlorhexidine digluconate (Slee & O‘Connor 1983, Decker et al. 2003). Tandjung et al (2007) investigated the antimicrobial activity of Octenisept on E.faecalis and concluded that it was particularly effective in dentin disinfection. According to the manufacturer (Schu¨lke & Mayr, Norderstedt, Germany), the toxicity parameters of Octenisept are well within compliant limits. No carcinogenic or mutagenic effects have been registered[36]. The

volume and contact time of the Octenisept irrigant was 2ml and 1 minute respectively to standardize with the irrigants used in the other groups. ***Till date no studies have reported the use of Octenisept in the elimination of fungi from the root canal system. Hence, Octenisept was chosen as an experimental irrigant in this study***.

The irrigation regimen used in groups IC and IIC was as follows: 2ml of 17% EDTA for one minute followed by 2ml of 5.25% NaOCl for one minute. A 5ml flush with distilled water to terminate the action of the irrigants and followed by 2ml of 1% Clotrimazole for one minute. It was hypothesized that the addition of an antifungal agent as an irrigant would provide a substantive action on the dentin and prevent adherence of C.albicans biofilm cells on the experimental samples. This was in accordance with the study by Saurabh et al (2010)[25] who evaluated the antifungal effect of 17% EDTA, 5.25% NaOCl and 2% CHX with and without an antifungal agent (Clotrimazole). One minute of contact time and the irrigant volume of 2ml was taken from the above study. In the present investigation, 1% Clotrimazole which is regularly used in the treatment of oral candidiasis was chosen.

Samples in groups ID and IID were irrigated with 2ml of Phosphate buffer saline for one minute and served as a control group.

Once the dentin disc samples were irrigated with the experimental irrigants, they were subjected to the Microbiological procedures. A suspension of C.albicans was adjusted to 0.5 turbidity on the Mcfarland scale. The canals of 64 experimental dentin disc samples were inoculated with 0.3ml of the adjusted C.albicans suspension, and each sample was individually submerged in Candida albicans suspension in the glass test tube vials. The samples were incubated at 36°c and 91% humidity for 72 hrs to form biofilms which simulates the environment inside the root canal system and resembles clinical situations. Out of the 10 dentin disc samples in each subgroup, 8 samples were assessed by the CFU method whilst the remaining 2 samples were assessed to detect the presence of Live/Dead Fungi in the dentinal tubules using the Confocal Laser Scanning Microscope. Hence, a total of 64 dentin disc samples were subjected to the CFU method and 16 dentin disc samples were used for the CLSM method.

CFU is a primary microbial technique, allowing determination of the number of viable fungi per sample. The samples analyzed by the CFU method were removed from the glass test tube vials and rinsed 3 times with 10 mL of sterile PBS. The root canal of each tooth sample was again enlarged with sterile #3 Gates-Glidden burs (0.9 mm diameter), and dentin shavings were collected into 3 mL of sterile PBS. The Gates-Glidden burs were also placed into the test tube to collect dentin shavings that adhered to the bur. All the tubes were sonicated in an ultrasonic water bath for 10 minutes to dislodge fungi from the burs and dentin shavings and to disperse fungal aggregation. Most studies evaluate root canal disinfection by sampling with paper points. This technique is limited by only sampling microbes from the fluid in the canal. In the present study, dentin shavings were sampled, which allowed the detection of fungi that had penetrated inside the dentinal tubules. This method realistically replicates the clinical scenario and highlights the degree to which C.albicans adhere to dentin and invade dentinal tubules following irrigation protocols. The antifungal efficacy was evaluated after 72hrs based on the number of colony forming units of Candida, which was obtained by semiquantitative analysis. This analysis was

adopted in this study because of its accuracy, reproducibility, acceptance and feasibility in the laboratory settings.

CLSM analysis determines the viable and dead fungi immobilized in the dentinal tubules and the biomass. The samples to be analyzed by the Confocal Laser Scanning Microscope were removed from the glass test tube vials and rinsed 3 times with 10 mL of sterile PBS. The samples were embedded on methyl methacrylate resin blocks and four evenly distributed transverse sections (1 mm thick) were cut using the Hard tissue microtome. The cut sections were then stained immediately with the SYTO 9 and propidium iodide (PI) reagents which are marketed as the Live/Dead stain (Baclight; Invitrogen Corporation, Carlsbad, CA, USA) and examined under the Confocal Laser Scanning Microscope.

The nucleic acid–binding fluors, SYTO 9 and propidium iodide (PI), have been widely applied in environmental studies, food microbiology and dental research including endodontic investigations[20]. These reagents were introduced by Invitrogen Corporation as the Baclight – Live/Dead stain, as they differentiate between viable and non-viable bacteria. However, Jin et al (2005)[9] evaluated the viability of candidal biofilms

using combination stains, SYTO9 and propidium iodide (PI) and demonstrated that SYTO9 and PI are reliable vital stains that may be used to investigate C. albicans biofilms under the Confocal Laser Scanning Microscope. Thus, the use of the fluorescent dyes to assess the viability of Candida albicans biofilms on the root canal dentin of young and old teeth has been confirmed in this study. SYTO 9 penetrates intact biological membranes, whereas PI penetrates only fungi with compromised plasma membranes and quenches the SYTO 9 fluorescence on binding the nucleic acid[11]. Thus, simultaneous application of the stains generates red-fluorescing dead fungi and green-fluorescing live fungi, and these can be visualized by fluorescence microscopy.

Confocal Laser Scanning Microscopy (CLSM) has become an invaluable tool for a wide range of investigations in the biological and medical sciences for imaging thin optical sections in living and fixed specimens ranging in thickness up to 100 micrometers[45]. Disinfection studies in the past have most commonly used the Scanning electron microscope (SEM) in the comparative assessment of antimicrobial endodontic irrigants and medicaments. Although, SEM evaluation can show the presence of total microorganisms on intratubular and

intertubular dentin, it fails to determine the viability of the immobilized organisms. Conventional microbiological staining methods have also been applied to dentinal sections to examine the distribution of microbes within the tubules but give no information on microbial viability. Recent studies in literature have revealed, that assessment of Viability of microorganisms following the use of disinfecting irrigating solutions directly correlates to the clinical effectiveness of the irrigants used[16]. The CLSM analysis on the other hand determines the viable and dead fungi immobilized in the dentinal tubules and is thus the appropriate tool of choice in this investigative study. Hence the CLSM method serves as a confirmatory guide and reflects the validity of the results obtained by the CFU method.

The results of the present study indicates that among the irrigants tested in the Young group, Octenisept was found to be most effective. This was followed by the combination of 17% EDTA + 5.25% NaOCl + 1% Clotrimazole. The irrigation regimen of 17% EDTA + 5.25% NaOCl (without the addition of the antifungal agent) ranked third. The specimens irrigated with PBS showed maximum candidal adherence to the dentin substrate and was the least effective. Similar results were

obtained when each of these irrigant combinations were used in the Old group of teeth.

The CFU counting results showed that irrigating the Octenisept solution or the addition of the antifungal agent to 17% EDTA + 5.25% NaOCl significantly reduced the Candida albicans adhesion in the root canals of both young and old groups. Control surfaces irrigated with Phosphate buffer saline indicated the highest amount of fungal adhesion in both age groups. Application of the 17% EDTA + 5.25% NaOCl combination also reduced the adhered fungi in the root canal but it was nowhere near effective as the two test irrigant groups used in the study. However, the inter group comparison revealed that ***the reduction in number of Candida albicans was significantly higher in the young group compared with the old group for all the irrigants used in the experiment.***

The CLSM evaluation results mirrored the results obtained by the CFU method and demonstrated more C.albicans in old root dentin as compared to the young root dentin. The two test irrigant combinations which performed best by the CFU analysis contained significantly fewer viable fungi in both age groups as evidenced by the scanty green fluorescence. Most

of the tubules were patent and empty with little or no fungal penetration. Control specimens treated with saline contained almost 94 – 96% of viable fungi in both the groups.

The results of this study indicates that higher amount of fungi are found in old root dentin as demonstrated by the CFU method and confirmed by the CLSM method. With increasing age, several histological changes occur in the dentin-pulp complex. Dentin sclerosis occurs as a result of an increase in peritubular dentin. Dentinal tubules become obliterated, resulting in narrowing of the tubule to approximately 2.5µm in diameter near the pulp and 0.9 µm in diameter near the enamel/cement[19]. In spite of the reduction in size with age, the tubule is still larger in diameter compared to the average C.albicans cell diameter of 1 – 1.5µm[33]. On the basis of these cellular dimensions it can be probable that fungi can attach and penetrate older dentinal tubules in spite of the obliteration phenomenon.

Dentin represents the primary substratum for candidal adhesion and biofilm formation in both primary and secondary infections of root canals[38]. Basically, dentin consists of an inorganic phase of apatite crystals and an organic matrix

primarily of collagen. Dentinal tubules contain appreciable amounts of unmineralized collagen[19]. It has been demonstrated that C.albicans adheres to collagen and maintains the capability to invade dentinal tubules[15]. There is limited understanding of the changes in the collagen matrix in dentin with aging. Ager et al reported that the amid 1 peak intensity of dentin collagen increased, whereas Nazari et al noted that collagen fibrils lose their extensibility depending on patient age[12]. These alterations in dentin collagen with aging might be one of the reasons for the differences of C.albicans adhesion capability to the root canal dentin observed in this study.

There is evidence that Candida has a special affinity for dentinal collagen and type I collagen significantly enhances candida adherence. The adherence of C. albicans to the extracellular matrix proteins, type I collagen and fibronectin is dependent upon the presence of extracellular calcium. This extracellular calcium is found to be abundant in old root dentin[33]. Venegas et al[40] reported that the adhesion of several types of bacteria to hydroxyapatite was enhanced with increasing Calcium ion concentration apart from the dentin surface. It was concluded that the higher mineral content in age-induced sclerotic dentin increased bacterial adhesion in old

root dentin. Taken together, the higher mineral content in age induced sclerotic dentin might be a contributory factor to the increased Candida albicans adhesion.

100% Octenisept was most effective against C.albicans in both age groups as per the results obtained by the CFU method and the same confirmed by the CLSM method. However, the older teeth showed a statistically significant increase in the number of fungi as compared to the young group. The efficacy of Octenisept was in accordance with the findings of Tirali et al[37] and Tandjung et al. The mode of action is fungicidal by interfering with cell walls and membranes. Octenidine hydrochloride was developed at the Sterling- Winthrop Research Institute as a potential topical antimicrobial agent. Previous studies with this compound have shown it to be effective in inhibiting the growth of plaque-forming bacteria and in reducing development of plaque in both experimental animals and humans. However, Its potential as an endodontic irrigant particularly against fungi has not been evaluated in the literature. It has been shown that octenidine resists an organic challenges, i.e. maintains its antimicrobial efficacy in the presence of organic material comparably to chlorhexidine and iodine (Pitten et al. 2003). This is of interest, as, in a root canal

system both organic and inorganic inhibitory factors are present that may weaken the antimicrobial efficacy (Haapasalo et al. 2000)[36]. The efficacy observed in the present study indicates the performance of octenidine was sufficient in this biologically complex environment as it exerts a substantive effect on dentin which blocks C.albicans biofilm colonization. However, the scope of the present study was only to evaluate the antifungal efficacy of Octenisept against C.albicans.

Among the irrigants used in the study, the next effective regime against the adherence of Candida albicans biofilms was the combination of 17% EDTA + 5.25% NaOCl + 1% Clotrimazole in both age groups as per the results obtained by the CFU method and confirmed by the CLSM method. However, the older teeth showed a statistically significant increase in the number of fungi as compared to the young group.

5.25% NaOCl was effective against C.albicans as shown by Radcliff et al[22], Vianna et al[41] in their studies. The antimicrobial effectiveness of sodium hypochlorite is based on its high pH (hydroxyl ions action). The high pH of sodium hypochlorite interferes with cytoplasmic membrane integrity by irreversible enzymatic inhibition, biosynthetic alterations in

cell metabolism and phospholipid destruction observed in lipidicperoxidation. The amino acid chloramination reaction forming chloramines interferes in cell metabolism. Oxidation promotes irreversible enzymatic inhibition of bacteria replacing hydrogen with chlorine. Enzyme inactivation can be observed in the reaction of chlorine with amino groups (NH_2) and an irreversible oxidation of sulphydryl groups (SH) of bacteria enzymes (cysteine). Thus, sodium hypochlorite presents antimicrobial activity with action on bacterial essential enzymatic sites promoting irreversible inactivation originated by hydroxyl ions and chloramination action[6]. Hence this could be the action of NaOCl to be effective against Calbicans.

17% EDTA was effective against C.albicans as shown by Sen et al[29], Mustafa et al in their studies. It has anticolonisation, anti-growth and anti-collagenolytic properties against C.albicans. By chelating calcium ions in the medium, EDTA prevents binding of C.albicans to the proteins in a dose-dependent manner. In the second process, EDTA reduces the growth of C.albicans by removing calcium from the cell walls and causing collapse in the cell wall and by inhibiting enzyme reaction[18]. Apart from its effective antifungal activity, EDTA plays a role in smear layer management. It opens the dentinal

tubules, which in turn allows deeper penetration of the medicaments and irrigants. Therefore, NaOCl and EDTA play a pivotal role in root canal disinfection.

1% Clotrimazole used as a final rinse following the universally used endodontic irrigants (EDTA and NaOCl) greatly augmented its antifungal effect against Candida albicans biofilm colonization on the root canal dentin in both age groups. This was in accordance to studies performed by Waltimo et al[43], Saurabh et al. The mechanism of action is by inhibition of the fungal cytochrome P450 enzyme to block demethylation of lanosterol 14-demethylase, which impairs the ergosterol synthesis leading to a cascade of membrane abnormalities in the Candida. This inhibition disrupts membrane function and increases permeability[25]. Clotrimazole is a relatively safe drug when used topically and also possesses antibacterial properties. Therefore, it can be used as an adjunct in root canal irrigation along with the already prescribed endodontic irrigants.

Following the two best performing irrigation protocols, the combination of 17% EDTA + 5.25% NaOCl (without the antifungal agent) ranked third against C.albicans in both age

groups as per the results obtained by the CFU method and confirmed by the CLSM method. However, the older teeth showed a statistically significant increase in the number of fungi as compared to the young group. The mechanism of action of this combination of irrigants is explained in detail above.

The least effective irrigant in this study was the Phosphate buffer saline which served as the control group in both age groups. However, the older teeth showed a statistically significant increase in the number of fungi as compared to the young group as per the results obtained by the CFU method and confirmed by the CLSM method. Based on its lack of antimicrobial effectiveness and current understanding of Candida pathophysiology it is not surprising to find that it had poor activity against fungi.

Candida albicans has been confirmed to have a strong association to failed endodontic cases which are refractory to treatment. Therefore, disinfection of the root canal during cleaning and shaping procedures should incorporate an antifungal agent to target the fungi specifically[14]. Each irrigant has a specific action and must be used in clinical endodontic practice to ensure complete disinfection. A combination of irrigants must be used to accomplish complete disinfection of

the root canal space. Although the use of the experimental irrigation regimens significantly decreased the biofilm of C.albicans, it did not totally eliminate all Candida in the root canals. This residue of alive or dead fungi attached on the surface might result in future reinfection of the root canal after chemomechanical preparation and hence assumes immense clinical significance. Because higher amounts of fungi were found in old root dentin, it can be concluded that in endodontic irrigation protocol, the volume and contact time of the irrigating solutions should be given due consideration. ***From the results of this investigation, the use of Octenisept or Clotrimazole can be recommended as a final rinse following the use of universal endodontic irrigants.***

In addition, a fluorescence microscopy method for the assessment of fungal viability and distribution within the mineralized tubules of ex vivo teeth has been adapted and validated. The technique provides a convenient and reproducible approach for assessing the viability of the fungi and the extent of fungal penetration into dentinal tubules. This technique will have application in the comparative assessment of antimicrobial effect of endodontic irrigants and medicaments in contemporary endodontics.

SUMMARY

The purpose of this study was to evaluate the effect of routine and newer endodontic irrigants on Candida albicans biofilm colonization in Young and Old human root canal dentin. Eighty intact mandibular premolars were used in this study and divided based on Age into two groups. Forty teeth were from young subjects (Group I) and the remaining 40 from old subjects (Group II). Dentin disc samples of 4mm were prepared from each tooth, standardized using gates glidden drill #2 and autoclaved for sterility. The forty samples each in the young and old group were divided into 4 subgroups with various irrigation protocols. The experimental irrigants were: A) 17% EDTA +5.25% NaOCl, B) 100% Octenisept, C) 17% EDTA +5.25% NaOCl + 1% Clotrimazole and D) Phosphate buffer saline. After the irrigation the experimental samples were inoculated with C.albicans and incubated for 72hrs. Out of the 10 samples in each subgroup, 8 samples were analyzed by the Colony forming unit method and 2 samples were analyzed by the Confocal laser scanning microscope. In the CFU method, aliquots from the experimental samples were plated on Sabouraud dextrose agar plates and the colony forming units were counted as a measure of antifungal activity. In the CLSM

method, fungal viability was demonstrated using special dyes SYTO 9 and Propidium iodide. The results showed that Octenisept was the most effective irrigant against C.albicans followed by addition of 1% Clotrimazole to 17% EDTA+5.25% NaOCl in both age groups. The other irrigant subgroups were less effective in both age groups. The results of this study also indicates that higher amount of fungi are found in old root dentin as demonstrated by the CFU method and confirmed by the CLSM method.

CONCLUSION

Under the limitations of this ex vivo study it can be concluded that:

1. Octenisept used as a single irrigant and the addition of an antifungal agent (1% Clotrimazole) to 17% EDTA + 5.25% NaOCl, were found to perform as the two best irrigation regimes among the irrigants tested in both age groups to reduce C.albicans adherence in root canal dentin.
2. Higher amount of fungi were found in old root dentin for all the irrigants tested as compared to the young group.
3. Confocal laser scanning microscopic evaluation to demonstrate fungal viability has been explored and confirmed in this investigation.

Further studies can be undertaken to investigate the use of Octenisept and Clotrimazole as a final rinse following routine endodontic irrigants in clinical trials against C.albicans.

REFERENCES

1. **Barcelos R, Gleiser R and Portela MB:**

   Inhibitory activity of root canal irrigants against Candida albicans, Enterococcus faecalis and Staphylococcus aureus. *Braz Oral Res. 2010; 24(4): 406-12.*

2. **Baumgartner C, Chad M.W, BS, Tian X:**

   Occurrence of Candida albicans in Infections of Endodontic origin.

   Journal of Endodontics, 2000; 26(12): 695-698.

3. **Brandle N, Zehnder M, Weiger R and Waltimo T:**

   Impact of Growth Conditions on Susceptibility of Five Microbial Species to Alkaline Stress.

   Journal of Endodontics, 2008; 34:579 –582.

4. **Carneiro M V, Juliana de M R and Antonio O C J:**

   Effect of Sodium Hypochlorite and Five Intracanal Medications on Candida albicans in Root Canals.

   Journal of Endodontics, 2001:27(6):401-403.

5. **Douglas J L:**

   Candida biofilms and their role in infection

   TRENDS in Microbiology 2003; 11(1).

6. **Estrela C, Estrela CRA, Barbin EL and Marchesa MA:**

   Mechanism of Action of Sodium Hypochlorite.

BrazDentJ, 2002; 13(2): 113-117.

7. **Ferguson J.W, Hatton J.Fand Gillespie M.J:**

Effectiveness of Intracanal Irrigants and Medications against the Yeast Candida albicans.

Journal of Endodontics, 2002; 28(2): 68-71.

8. **Jill R and Mitchell A:**

How to build a biofilm: a fungal perspective.

Current Opinion in Microbiology 2006, 9:588–594

9. **Jin Y, Zhang T, Samaranayake Y.H and Yip H.K:**

The use of new probes and stains for improved assessment of cell Viability and extracellular polymeric substances in Candida albicans biofilms.

Mycopathologia 2005; 159: 353–360.

10. **Kakoli P, Nandakumar R, Romberg E and Arola D:**

The Effect of Age on Bacterial Penetration of Radicular Dentin.

Journal of Endodontics, 2009; 35:78–81.

11. **Kania R.E, Herman P, Hiemstra P and Frijns J:**

Biofilms on tracheoesophageal voice prostheses: a confocal laser scanning microscopy demonstration of mixed bacterial and yeast biofilms.

Biofouling 2010; 26(5):519–526.

12. Kinney J.H, Nalla R.K, Pople J.A and Ritchie R.O:

Age-related transparent root dentin: mineral concentration, crystallite size, and mechanical properties.

Biomaterials 2005; 26:3363–3376.

13. Lamfon H, Porter SR and Pratten J:

Formation of Candida albicans biofilms on non-shedding oral surfaces.

Eur J Oral Sci 2003; 111: 465–471.

14. Lau H, Ballal V, Shenoy S and Acharya S:

Evaluation of antifungal efficacy of 5% doxycycline hydrochloride, 2.5% sodium hypochlorite, 17% ethylenediamine tetraacetic acid and 0.2% chlorhexidine gluconate against candida albicans - An in vitro study.

Journal of Endodontology, 2010; 6-13.

15. Love R M:

Invasion of dentinal tubules by root canal bacteria.

Endodontic Topics 2004; 9: 52–65.

16. Ma Jingzhi, Wang Z and Haapasalo M:

A New Noninvasive Model to Study the Effectiveness of Dentin Disinfection by Using Confocal Laser Scanning Microscopy.

Journal of Endodontics, 011; 37:1380–1385.

17. Molander A, Reit C, Dahlen G, and Kvist T:

Microbiological status of root-filled teeth with apical periodontitis.

International Endodontic Journal, 1998; 31: 1-7.

18. Mustafa A, Akdeniz BG, Sen BH and Izmir:

The effect of calcium chelating or binding agents on Candida albicans.

Oral Surg Oral Med Oral Pathol Oral Radiol Endod, 2005; 100(5): 626-63.

19. Ozdemir H.O, Buzoglu H.D, Calt S and Steinberg D:

Effect of Ethylenediaminetetraacetic Acid and Sodium Hypochlorite Irrigation on Enterococcus faecalis Biofilm Colonization in Young and Old Human Root Canal Dentin: In Vitro Study.

Journal of Endodontics, 2010; 36:842–846.

20. Parmar D, Hauman C.H.J, Leichter J.W and Tompkins G.R:

Bacterial localization and viability assessment in human ex vivo dentinal tubules by fluorescence confocal laser scanning microscopy.

International Endodontic Journal, 2011; 44:644–651.

21. Peciuliene V, Reynaud A.H, Balciuniene I and Haapasalo M:

Isolation of yeasts and enteric bacteria in root-filled teeth with chronic apical periodontitis.

International Endodontic Journal, 2001; 34: 429-434.

22. Radcliffe C.E, Potouridou L and Drucker D.B:

Antimicrobial activity of varying concentrations of sodium Hypochlorite on the endodontic microorganisms Actinomyces israelii, A.naeslundii, Candida albicans and Enterococcus faecalis.

International Endodontic Journal, 2004; 37: 438-446.

23. Richards D, Davies JK and Figdor D:

Starvation survival and recovery in serum of Candida albicans compared with Enterococcus faecalis.

Oral Surg Oral Med Oral Pathol Oral Radiol Endod 2010; 110: 125-130.

24. Ruff ML, Mcclanahan SB and Britta SB:

In Vitro Antifungal Efficacy of Four Irrigants as a Final Rinse.

Journal of Endodontics, 2006; 32(4): 331-333.

25. Saurabh S.Chandra, M.R Srinivasan and Rajamani Indira:

Antifungal Efficacy of 5.25% Sodium Hypochlorite, 2%

Chlorhexidine Gluconate, and 17% EDTA With and Without an Antifungal Agent.

Journal of Endodontics 2010;36: 675–678.

26. Sen BH, Safavi KE and Spangberg LSW:

Colonization of Candida Albicans on cleaned human dental hard tissues.

Archs oral Biol. 42(7):513-520.

27. Sen BH, Safavi KE, Spangberg LSW, Ege and Farmington:

Growth patterns of Candida albicans in relation to radicular dentin.

Oral Surg Oral Med Oral Pathol Oral Radiol Endod, 1997; 84: 68-73.

28. Sen BH, Safavi KE and Spangberg LSW:

Antifungal Effects of Sodium Hypochlorite & Chlorhexidine in Root canals.

Journal of Endodontics, 1999; 25(4): 235-238.

29. Sen BH, Akdeniz G, Denizci A and Izbir:

The effect of ethylenediamine-tetraacetic acid on Candida albicans.

Oral Surg Oral Med Oral Pathol Oral Radiol Endod ,2000; 90(5): 651-655.

30. Sen BH, Chugal NM, Calif and Izmir:

A new method for studying the adhesion of Candida albicans to dentin in the presence or absence of smear layer.

Oral Surg Oral Med Oral Pathol Oral Radiol Endod, 2003; 2: 201-206.

31. Semwogerere D and Weeks ER:

Confocal microscopy.

Encyclopedia of Biomaterials and Biomedical Engineering 2005; 1-10.

32. Sena NT, Vianna ME, Berber VB and Zaia AA:

In vitro antimicrobial activity of sodium hypochlorite and chlorhexidine against selected single-species biofilms.

International Endodontic Journal 2006; 39: 878–885.

33. Siqueira JF, Rocas IN, Lopes HP, Elias CN and Milton de U:

Fungal Infection of the Radicular Dentin.

Journal of Endodontics, 2002; 28(11): 770-773.

34. Siqueira JF, Rocas IN and Lopes HP:

Elimination of Candida albicans Infection of the Radicular Dentin by Intracanal Medications.

Journal of Endodontics, 2003; 29(8): 501-504.

35. Siqueira JF, Sen BH, Rio de J and Izmir:

Fungi in endodontic infections.

Oral Surg Oral Med Oral Pathol Oral Radiol Endod, 2004; 97: 632-641.

36. Tandjung L, Waltimo T, Hauser I, Heide P and Weiger R: Octenidine in root canal and dentine disinfection ex vivo. *International Endodontic Journal 2007; 40: 845–851.*

37. Tirali R, Turan Y and Akal N:

In vitro antimicrobial activity of several concentrations of NaOCl and Octenisept in elimination of endodontic pathogens.

Oral Surg Oral Med Oral Pathol Oral Radiol Endod 2009; 108:117-120.

38. Turk T, Ates M, Sen BH and Izmir:

The effect of treatment of radicular dentin on colonization patterns of *C. albicans.*

Oral Surg Oral Med Oral Pathol Oral Radiol Endod 2008; 106:457-62.

39. Turk T, Sen BH and Ozturk T:

In vitro antimicrobial activity of calcium hydroxide mixed with different vehicles against Enterococcus faecalis and Candida albicans.

Oral Surg Oral Med Oral Pathol Oral Radiol Endod 2009; 108:297-301.

40. Venegas SC, Palacios JM, Apella MC, Morando PJ and Blesa MA:

Calcium modulates interactions between bacteria and hydroxyapatite.

J Dent Res 2006; 85:1124–8.

41. Vianna ME, Gomes B, Berber VB, Zaia AA and Randi FC:

In vitro evaluation of the antimicrobial activity of chlorhexidine and sodium hypochlorite.

Oral Surg Oral Med Oral Pathol Oral Radiol Endod, 2004; 97(1): 79-84.

42. Waltimo TMT, Orstavik D, Siren EK and Haapasalo MPP:

In Vitro Yeast Infection of Human Dentin.

Journal of Endodontics, 2000; 26(4): 207-209.

43. Waltimo TMT, Orstavik D, Meurman JH and Haapasalo MPP:

In vitro susceptibility of Candida albicans isolates from apical and marginal periodontitis to common antifungal agents.

Oral Immunology, 2000; 15(4): 245-248.

44. Waltimo TMT, Sen BH, Orstavik D and Haapasalo MPP:

Yeasts In Apical Periodontitis.

CritRev Oral Biol Med, 2003; 14(2): 128-137.

45. White JG and Amos WB:

How the confocal laser scanning microscope entered biological research.

Biology of the Cell 2005; 95: 335–342.

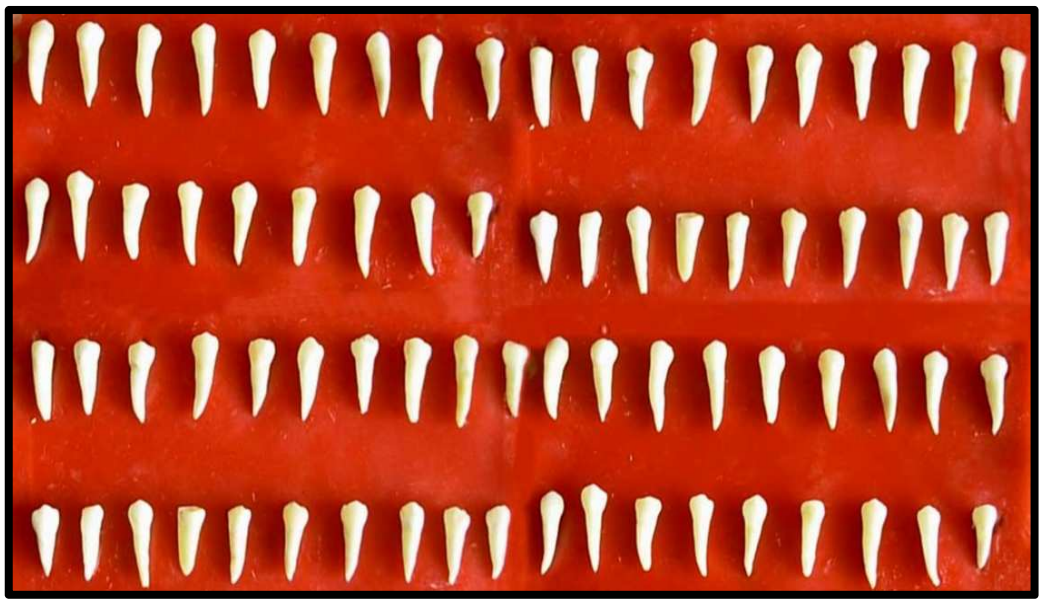


Fig 1: Tooth Samples (Mandibular Premolars)

Fig 2: Decoronation at CEJ level

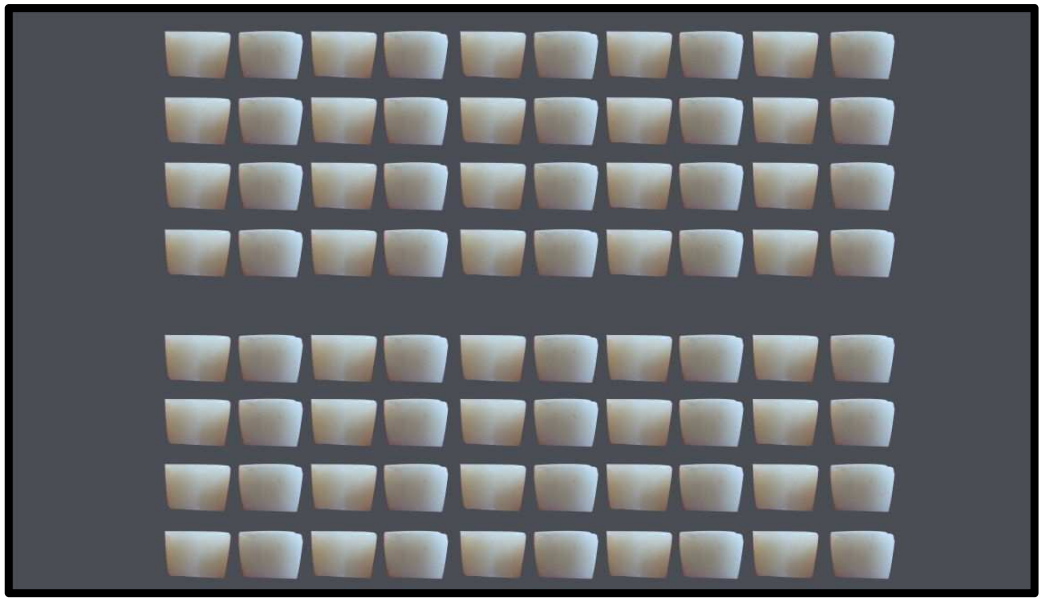


Fig 3: Dentin Disc Samples

Fig 4: Materials and Armamentarium

Fig 5: Mc Farland Scale

Fig 6: Autoclave

Fig 7: Sabouraud dextrose Agar

Fig 8: Sabouraud dextrose Broth

Fig 9: Experimental Irrigants

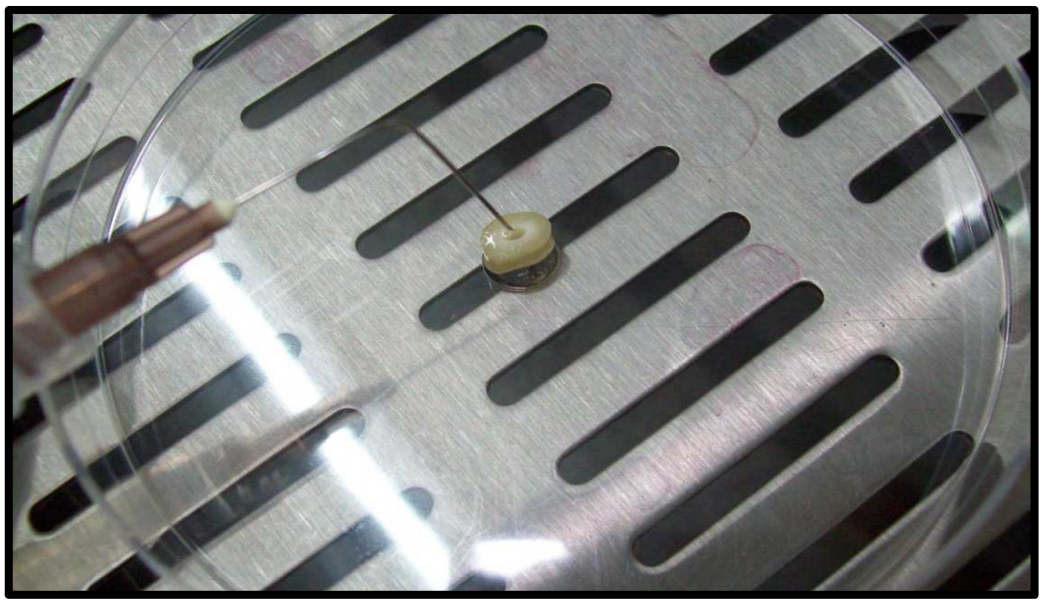


Fig 10: Irrigating the Specimen

Fig 11: Inoculation with C.albicans

Fig 12: Samples incubated at 36°C for 72 hrs in Incubator

Fig 13: Collection of Samples

Fig 14: Streaking of aliquot

SEVENTY TWO HOURS AFTER STREAKING

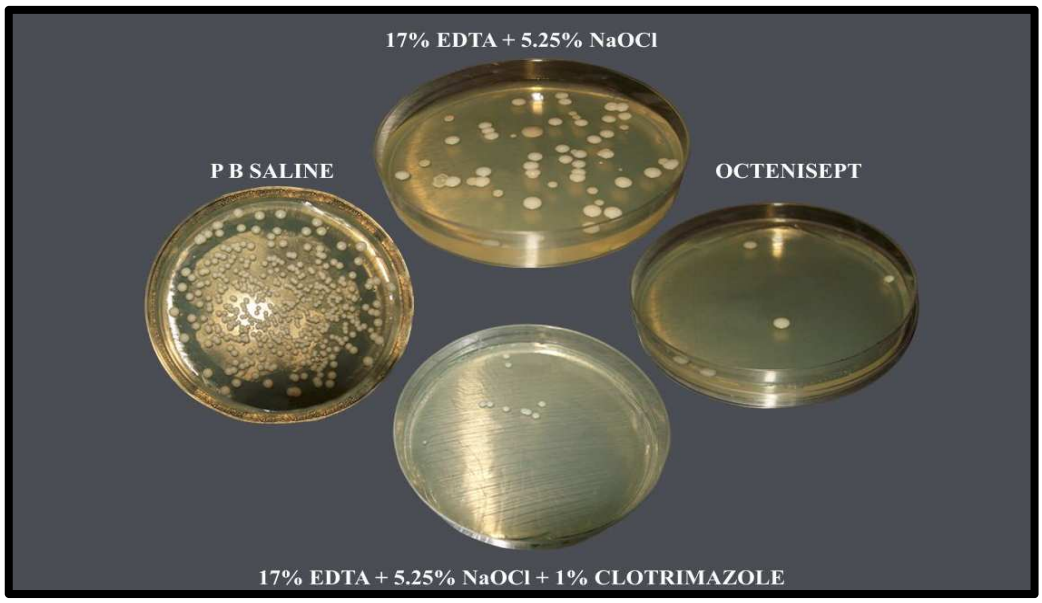
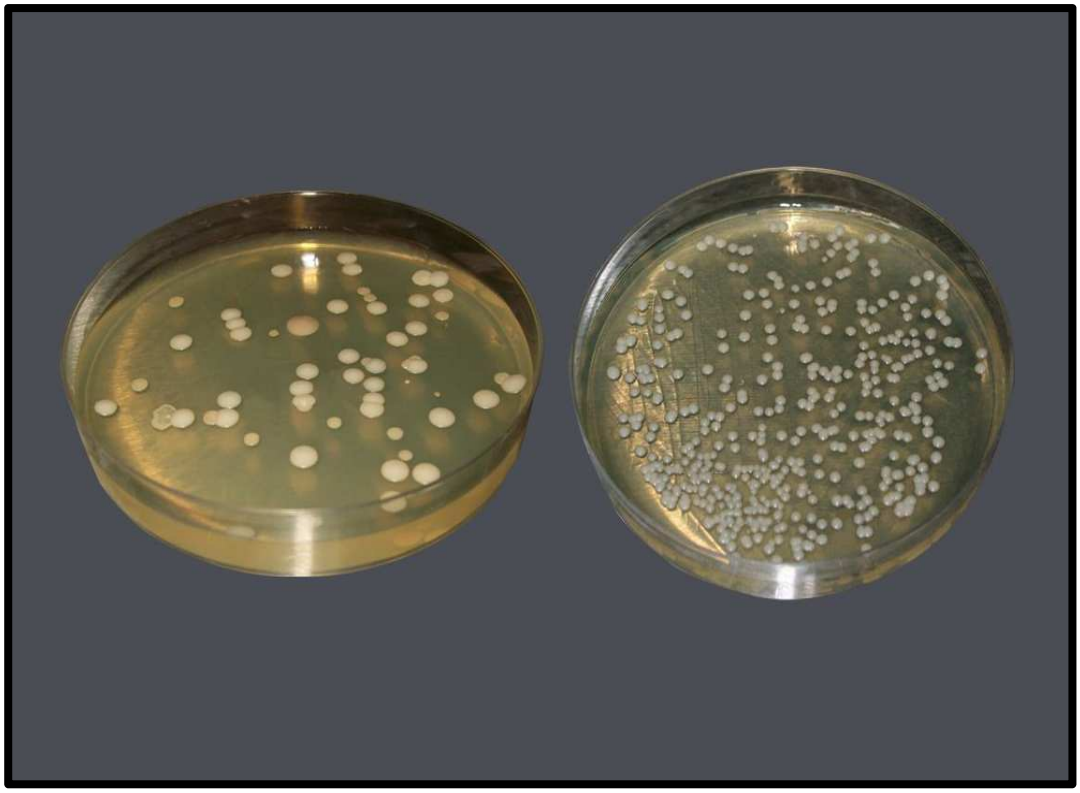


Fig 15: Group I (Young Teeth)

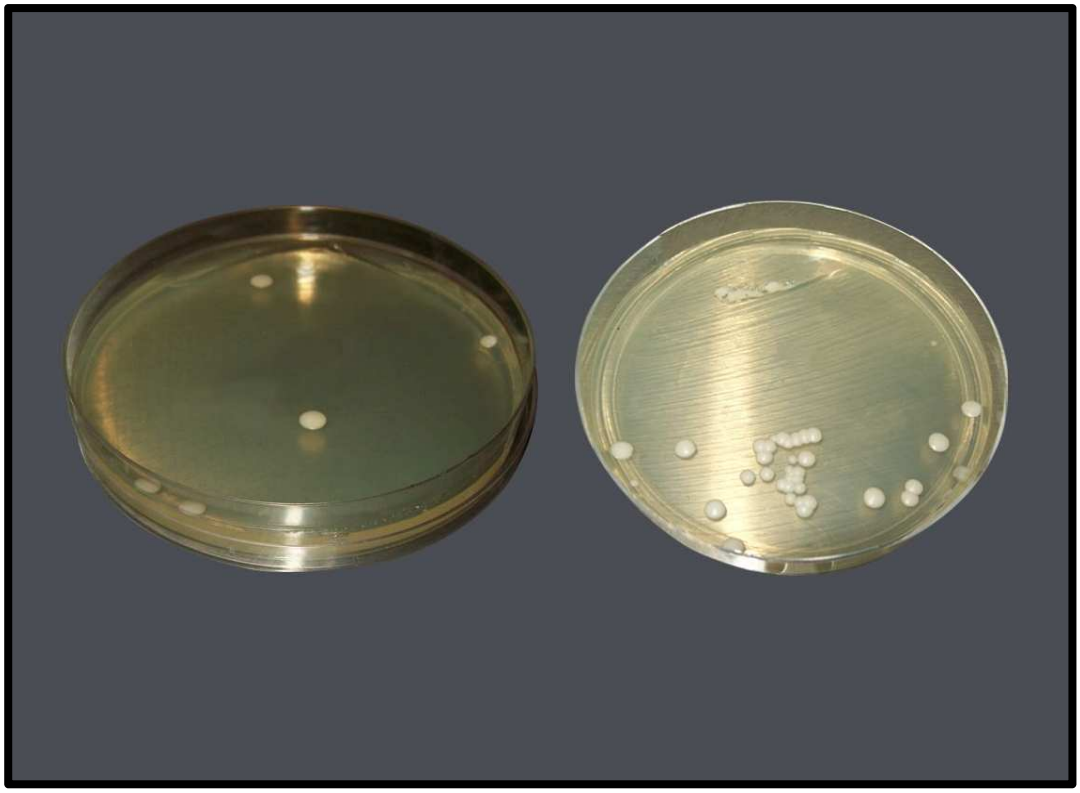
Fig 16: Group II (Old Teeth)

Fig 17: C.F.U WITH 17% EDTA + 5.25% NaOCl



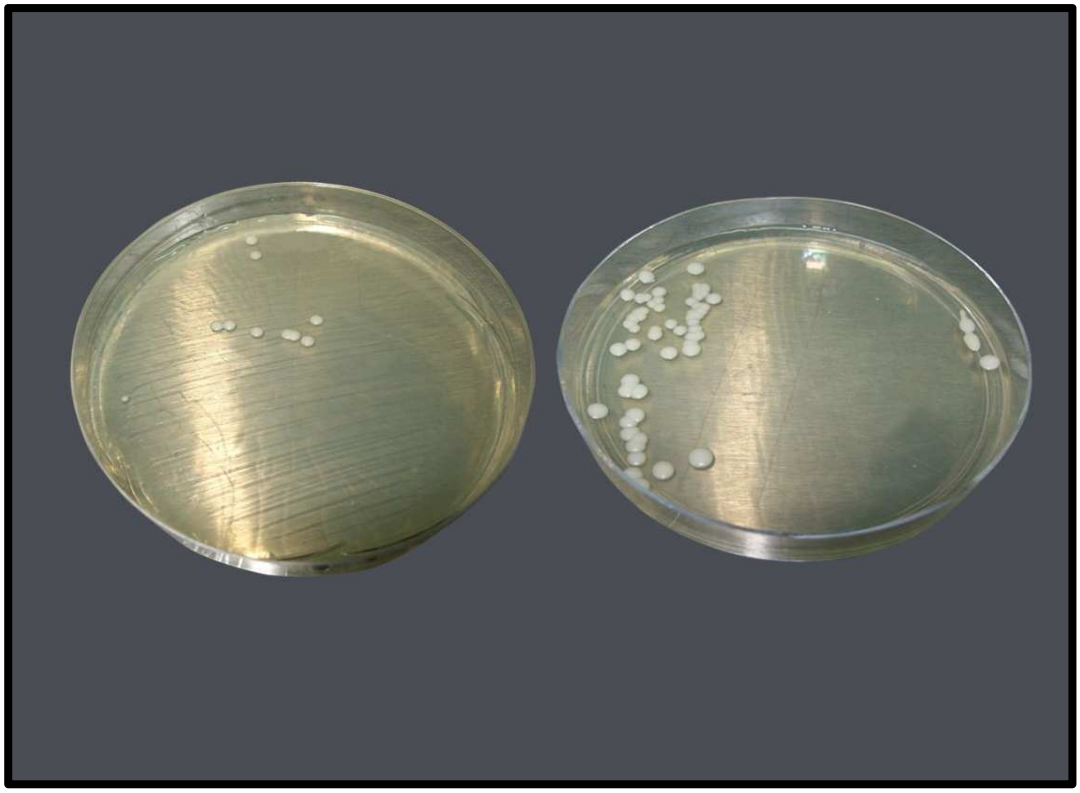
Young (IA) **Old (IIA)**

Fig 18: C.F.U WITH OCTENISEPT



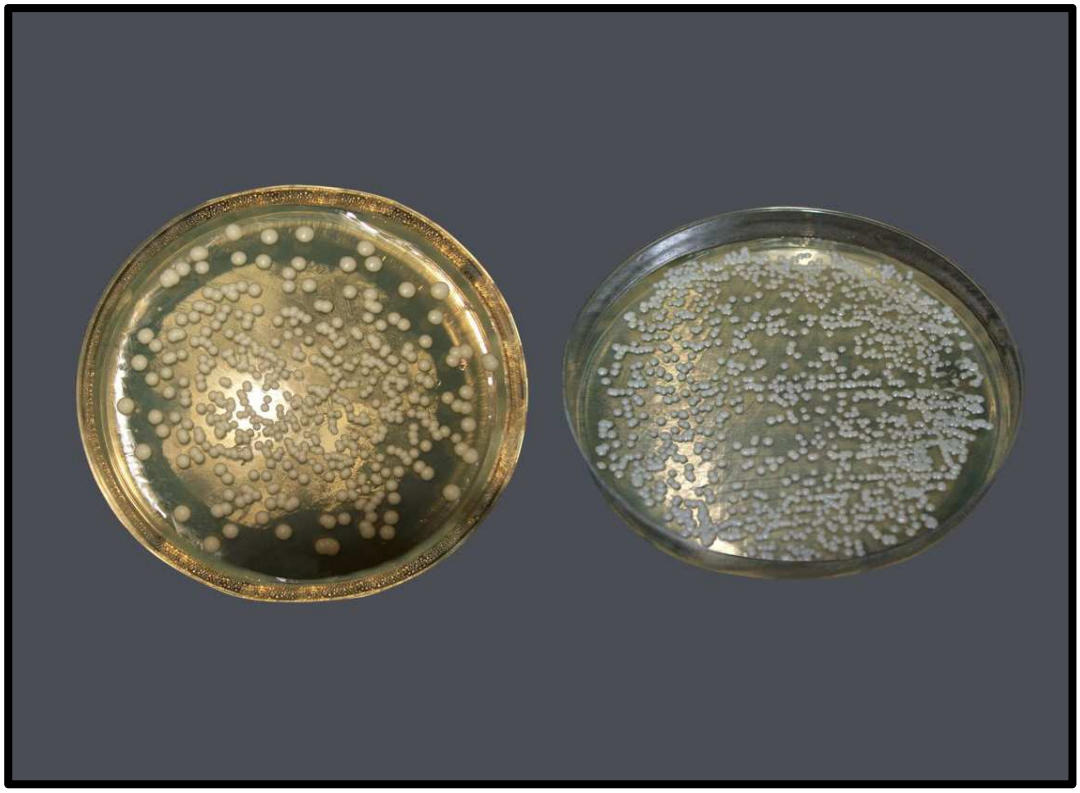
Young (IB) **Old (IIB)**

Fig 19: C.F.U WITH 17% EDTA + 5.25% NaOCl + 1% CLOTRIMAZOLE



Young (IC) **Old (IIC)**

Fig 20: C.F.U with Phosphate Buffer Saline



Young (ID) **Old (IID)**

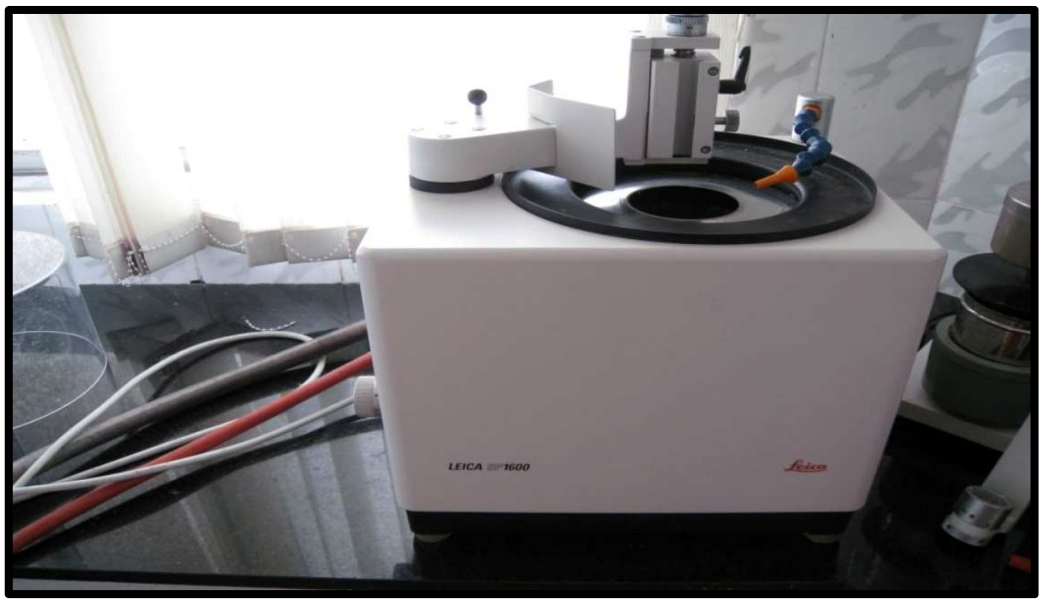


Fig 21: Hard Tissue Microtome

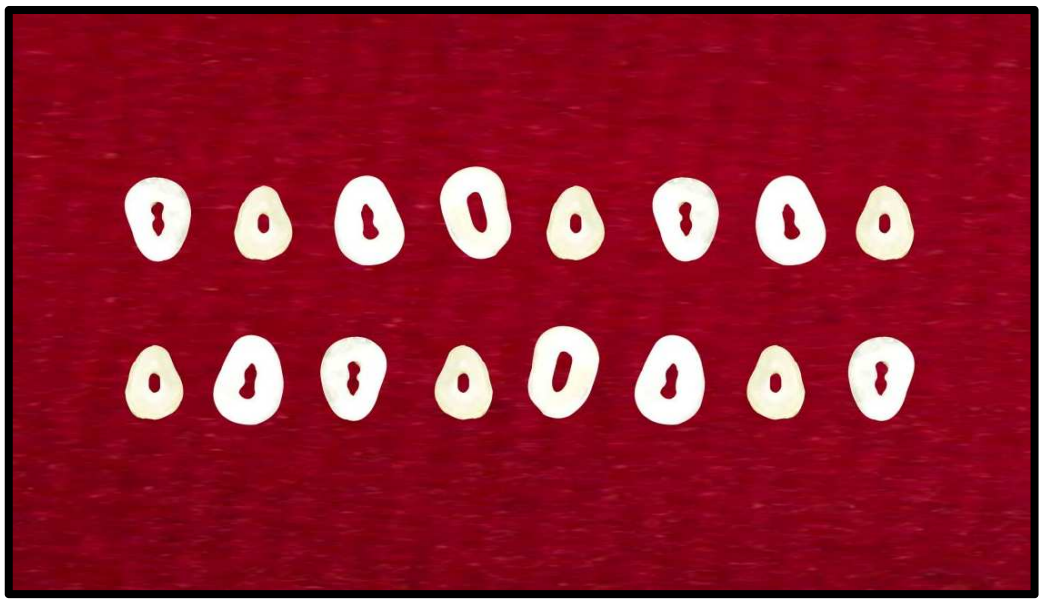


Fig 22: Teeth Samples (1mm Cross Section)

Fig 23: Bactec Viability Kit (INVITROGEN)

Fig 24: Staining with SYTO 9 and Propidium Iodide Dyes

Fig 25: Confocal Laser Scanning Microscope

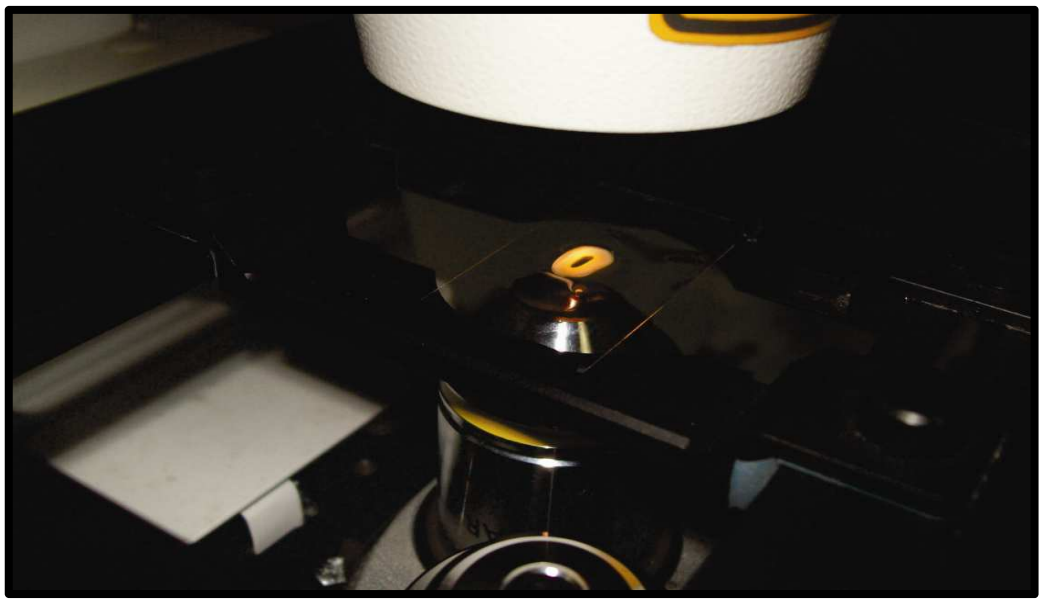
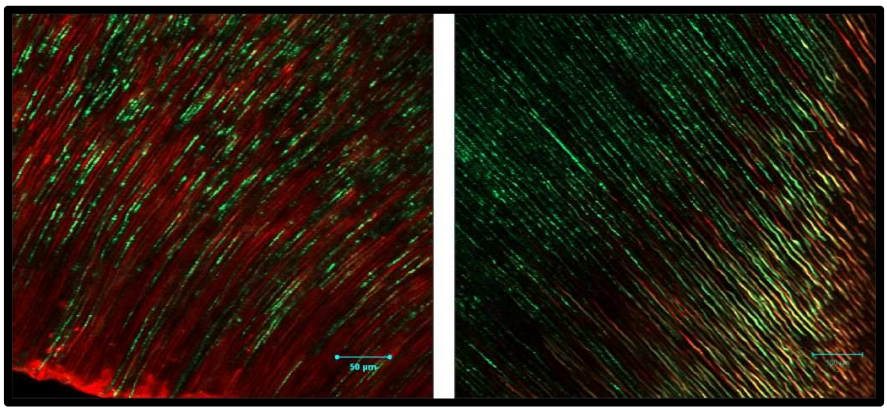


Fig 26: Sample Scanning Examination

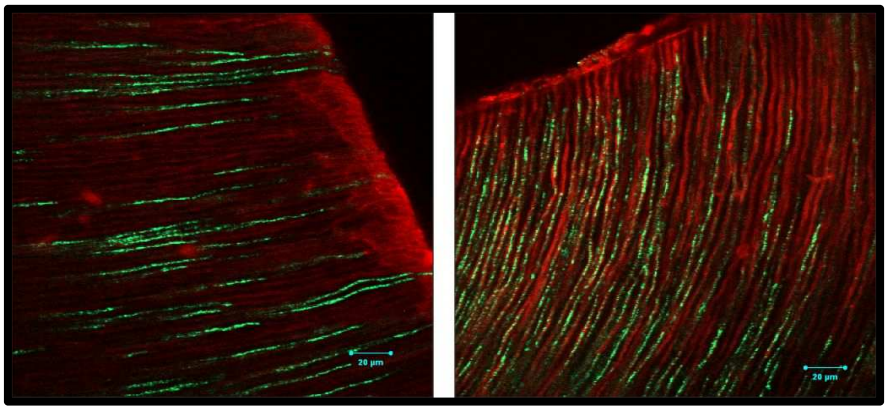
Fig 27: Sub Group A: (17% EDTA + 5.25% NaOCl)

YOUNG (IA) **OLD (IIA)**

20X



40X



63X

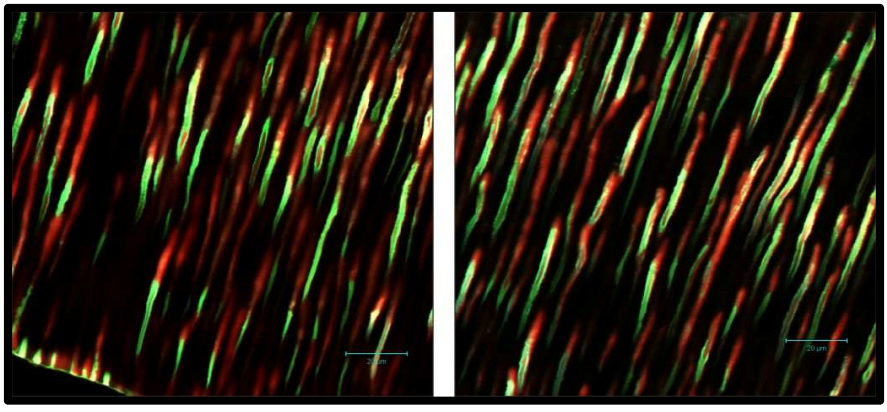
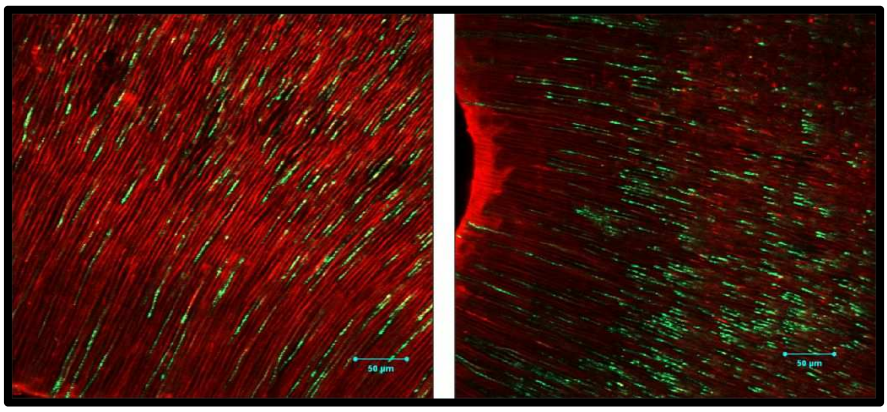


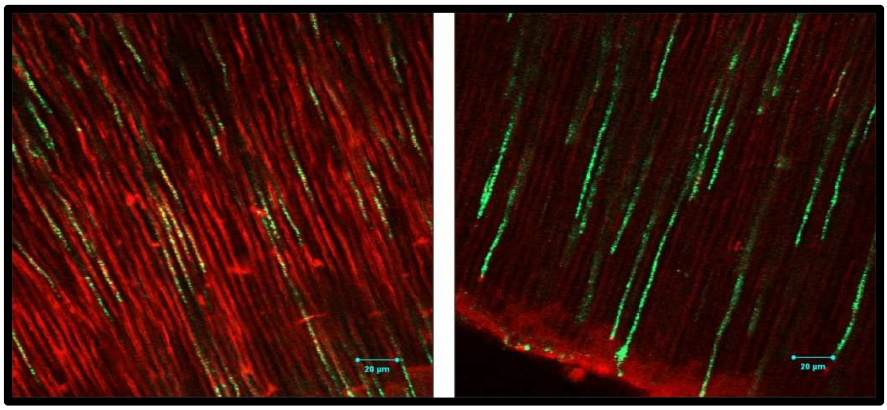
Fig 28: Sub Group B: (OCTENISEPT)

YOUNG (IB) **OLD (IIB)**

20X



40X



63X

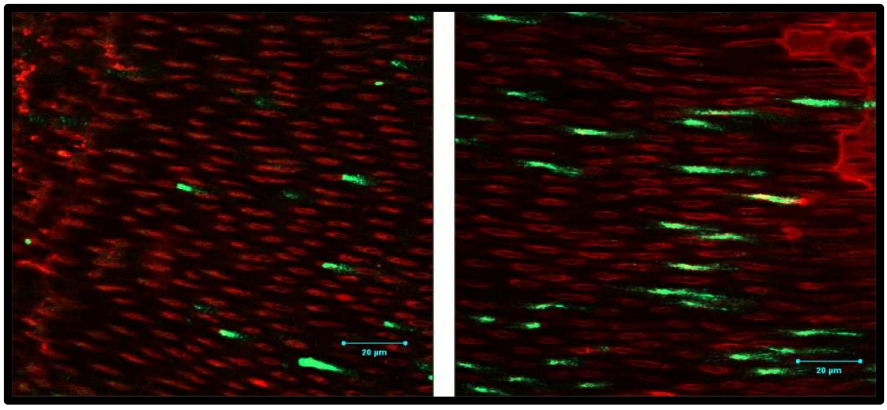
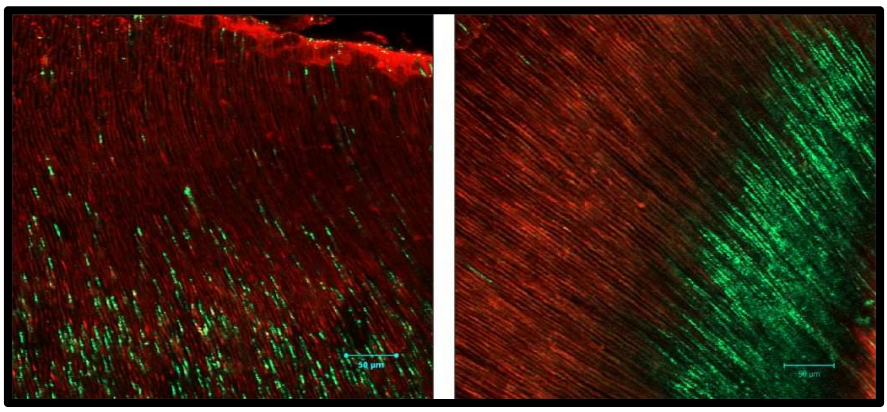


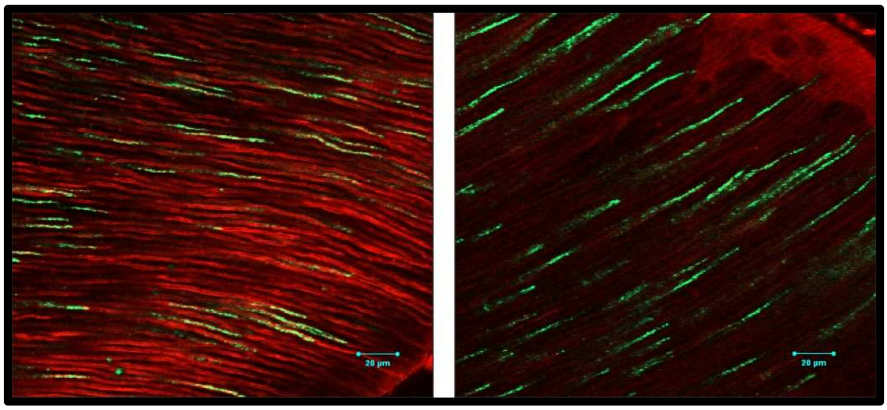
Fig 29: Sub Group C: (17% EDTA + 5.25% NaOCl + 1% CLOTRIMAZOLE)

YOUNG (IC) OLD (IIC)

20X



40X



63X

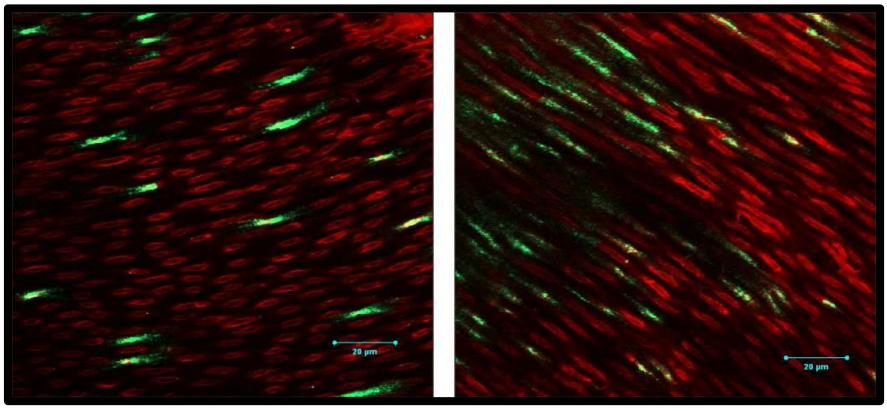
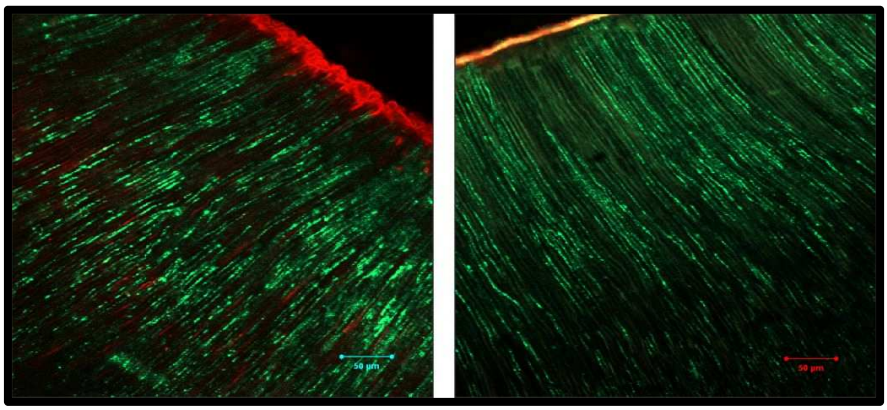


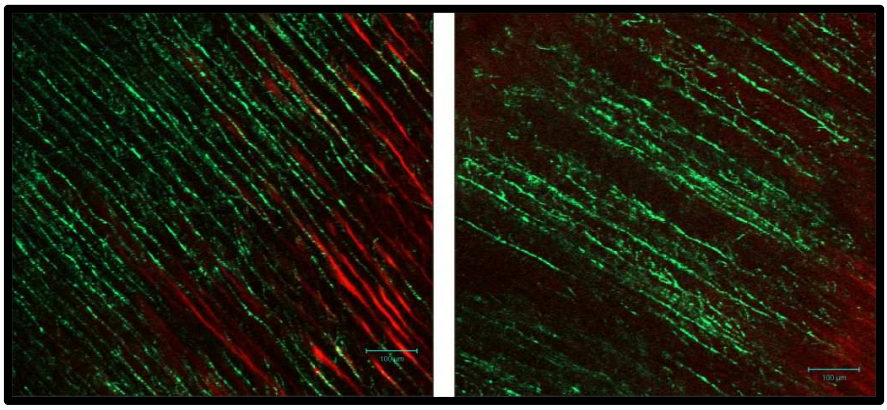
Fig 30: Sub Group D: (PHOSPHATE BUFFER SALINE)

YOUNG (ID) **OLD (IID)**

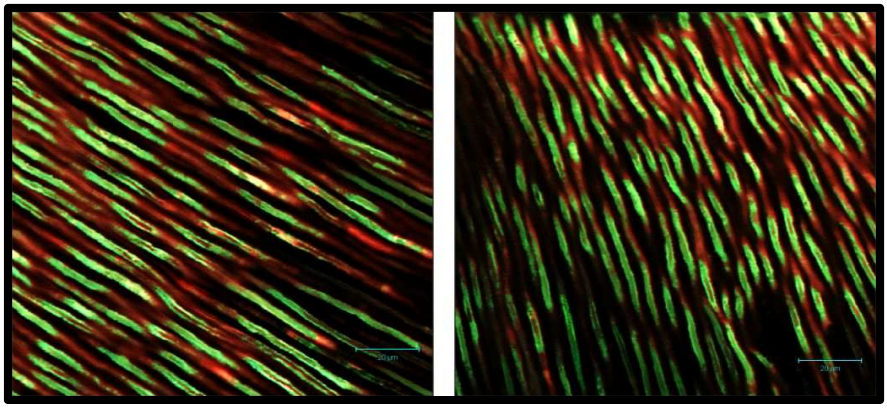
20X



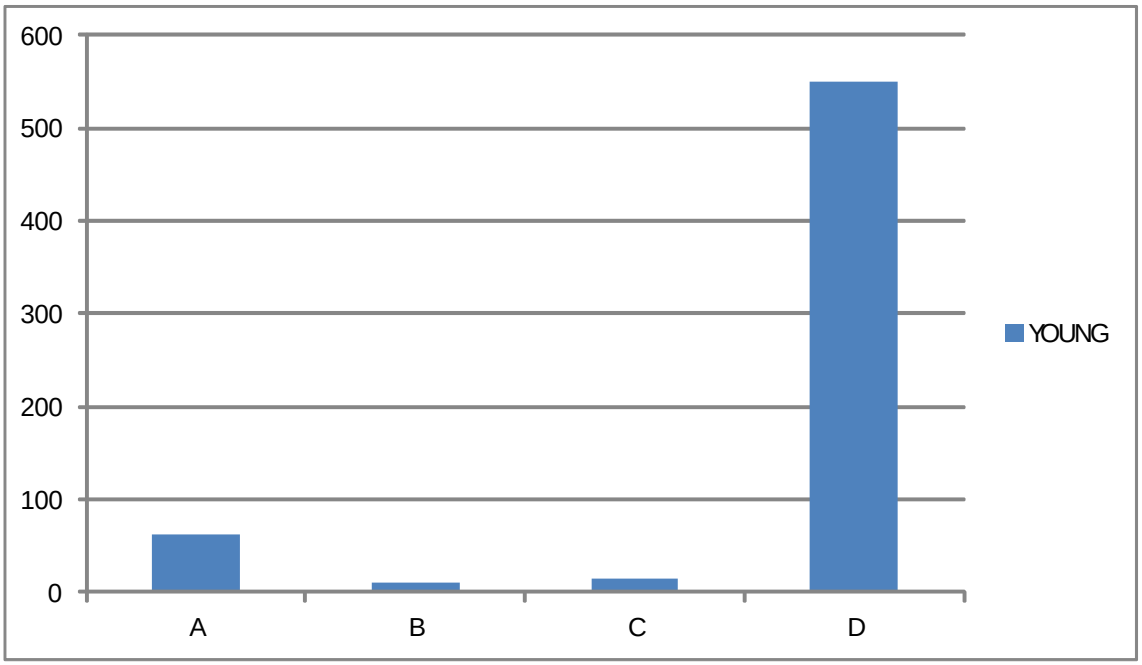
40X



63X



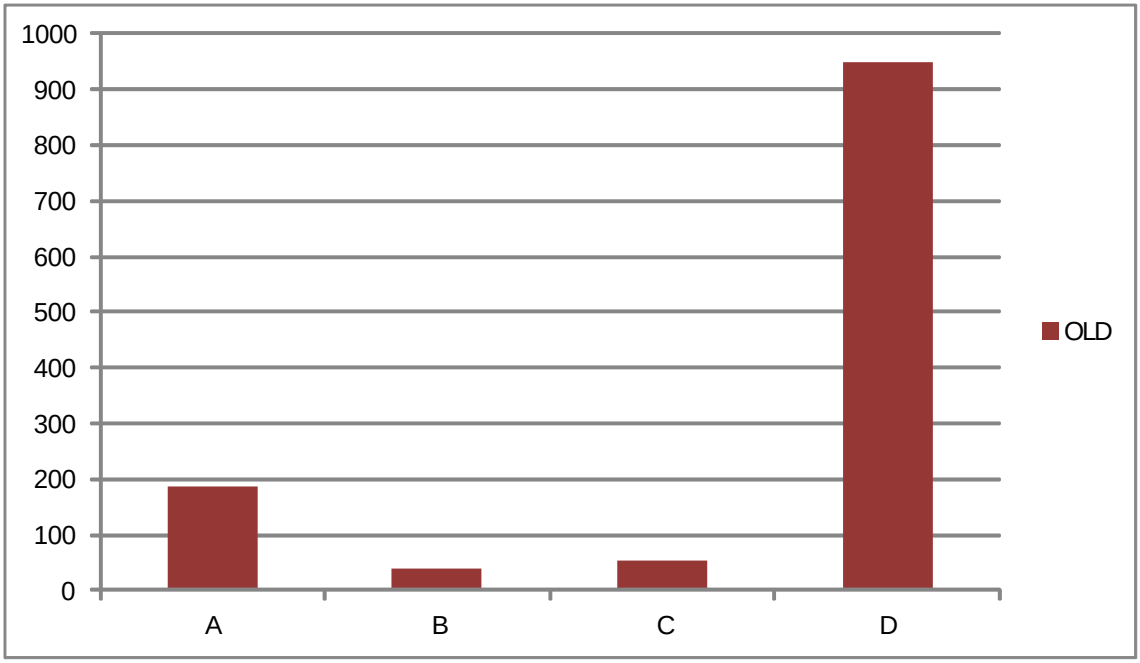
COMPARISON OF IRRIGANTS IN YOUNG GROUP



C F U

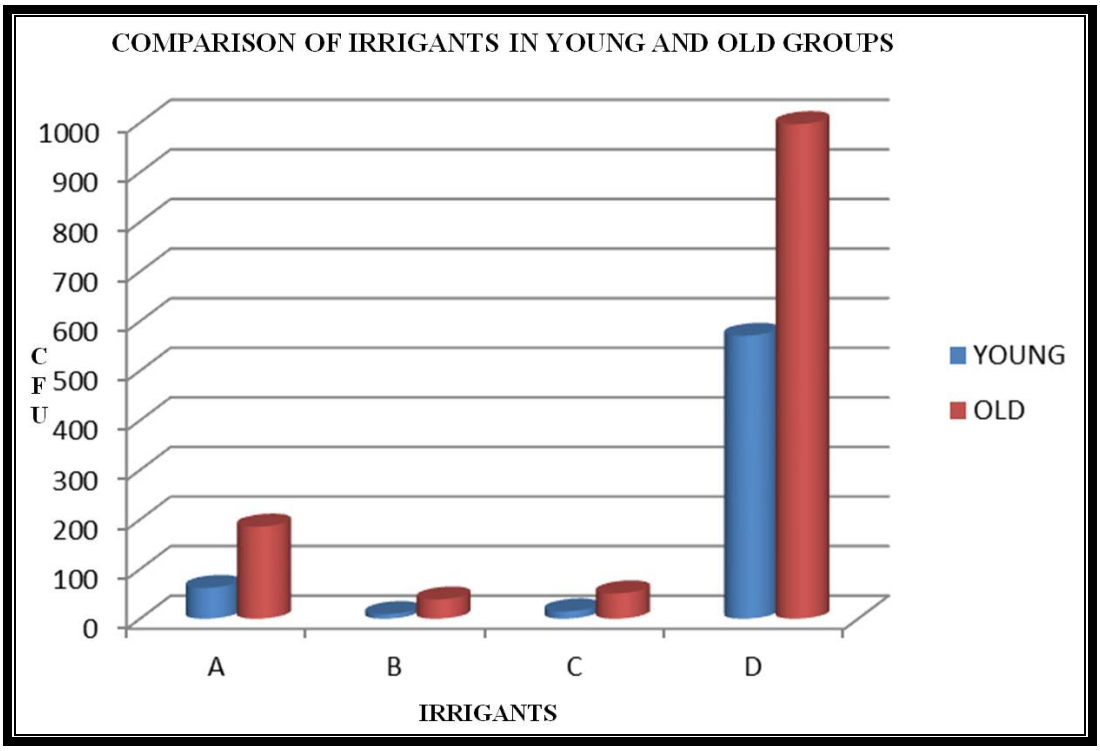
IRRIGANTS

COMPARISON OF IRRIGANTS IN OLD GROUP



C F U

IRRIGANTS



A. 17% EDTA + 5.25% NaOCl

B. Octenisept

C. 17% EDTA + 5.25% NaOCl + 1% Clotrimazole

D. Phosphate Buffer Saline